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Effect of amylin and salmon calcitonin on energy expenditure

INAUGURAL DISSERTATION

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1 Summary

In the present study, we investigated the acute and chronic effects of amylin and its agonist salmon calcitonin (sCT) on energy expenditure in rats. The anorectic effect of amylin on food intake is well known. However, the role of amylin in the control of energy output is less clear. Based on evidence of an increase in body temperature and a body-weight loss after amylin or sCT application, we wanted to test the hypothesis that amylin and sCT increase energy expenditure.

Our investigations resulted in the following findings:

- At a dose, that significantly reduced food intake, a single injection of amylin did not affect energy expenditure under our conditions.

- SCT, which is more potent than amylin due to sCT's irreversible binding to the amylin receptor, significantly increased energy expenditure when rats had no access to food after an acute injection.

- After a chronic infusion of amylin, energy expenditure tended to be increased compared to the yoked group, but no effect was observed compared to the saline group.

We conclude that amylin may prevent the decrease in energy expenditure that is normally seen in animals that eat less. Further, the significant increase in energy expenditure after an acute sCT injection suggests that sCT has a stronger effect on energy expenditure than amylin,. A chronic infusion of amylin may also prevent the

decrease in energy expenditure that would normally occur as a result of reduced food intake.

2 Introduction

2.1 Energy balance

A balance between energy intake and energy expenditure results in a constant body weight. These two factors depend on various components like food intake, physical activity, basal metabolism, and thermoregulation. Food intake in particular also depends on emotions, social factors, habit, time of day and convenience. The latter variables are not part of the homeostatic mechanisms that control energy balance, but nonetheless affect meal-to-meal energy intake. As a consequence, daily energy intake is variable both within and between individuals, and it is not necessarily well correlated with energy expenditure on a day to day basis. If energy intake exceeds the energy expenditure for a longer period, the excess of energy will be stored as body fat which will finally result in obesity. On the other hand, weight-loss can only be reached if energy expenditure exceeds energy intake. Despite short-term mismatches in energy balance, most people match cumulative energy intake to energy expenditure with remarkable precision when measured over a period that spans many meals.

Different hormones play an important role in the long-term control of energy balance. The pancreatic hormone insulin, which reaches the brain via the circulation acts in the nucleus arcuatus and it reduces energy intake. Insulin was the first hormonal signal to be implicated in the control of body weight by the central nervous system (CNS) (Woods et al. 1979). The demonstration that the profound hyperphagia and obesity of ob/ob mice, resulting from an autosomal recessive mutation of the gene encoding for leptin, provided compelling evidence of a second adiposity signal (Zhang et al. 1994). Insulin and leptin circulate at levels proportional to body fat and

enter the CNS in proportion to their plasma levels (Bagdade et al. 1967; Considine et al. 1996; Schwartz et al. 1996). Chronic leptin replacement has been demonstrated to cause a marked reduction in food consumption, loss of body weight, elevation of body temperature and increase in energy expenditure in ob/ob mice (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995). Single injection of human leptin had a profound effect on whole body energy expenditure in ob/ob mice (Hwa et al. 1997).

Recent studies provided evidence that amylin may be a third important hormone involved in long term control of energy balance.

2.2 Amylin

2.2.1 Structure, synthesis and secretion

Amylin, also known as islet amyloid polypeptide (IAPP), belongs to the family of the calcitonin gene peptides. All these peptides, i.e. calcitonin, calcitonin gene related peptide (CGRP), amylin and adrenomedullin, have overlapping biological effects owing to similar structures and cross-reactivity between receptors (Wimalawansa 1997). Amylin is a 37-amino acid peptide with a molecular mass of about 3.9 kD (Cooper et al. 1987). It is a normal secretory product of pancreatic β-cells, which constitute amylin's main production site. Some amylin synthesis also seems to occur in the gastrointestinal tract and spinal ganglia (Mulder et al. 1997).

Amylin is mainly stored in the β-cell granules of the pancreas (Johnson et al. 1988) and it is co-secreted with insulin after glucose or mixed-meal ingestion (Butler et al. 1990). The release of these two hormones occur at a relatively constant ratio of about 1:100 (amylin : insulin) in non-diabetic subjects (Ludvik et al. 1991; Hull et al.

2004). Insulin has a short half-life ($t_{1/2}$) of less than 5 minutes compared with amylin . Amylin is cleared through the kidney resulting in a longer $t_{1/2}$ of about 15 min (Hull et al. 2004). Therefore, due to the slower plasma clearance rate of amylin, the ratio of amylin and insulin in fasted individuals arises to 1:10.

2.2.2 Regulation of food intake by amylin

The molar functional unit of eating is a meal. Therefore, eating has to be analyzed in terms of the controls of meal initiation (usually called hunger), maintenance of eating during the meal (one aspect of food reward), meal termination (satiation), and inhibition of eating following meals (postprandial satiety). For most of us, the impetus to begin a meal is not based on a biological deficit or need such as insufficient glucose or other energy source in some critical tissue. In fact, little is known about the physiological controls of meal initiation, maintenance of a meal and postprandial satiety. Signals leading to meal termination are comparatively well investigated. One of these signals is amylin.

Early experiments indicated that amylin shares its effect to reduce food intake with other peptides of the calcitonin gene peptide family (Chance et al. 1991). A number of studies demonstrated amylin's anorectic effect after peripheral injection. In an early study with 12-h food-deprived old (15–18 month) rats, food intake was significantly decreased by amylin (1-10 μg/kg) when injected i.p. at the beginning of the dark phase. The anorectic effect was most marked in the first 2h after amylin injection and was compensated over 24h. In young (7-9 weeks) rats, amylin (0.1-1 μg/kg) dose-dependently reduced food intake in rats that were food-deprived for 24h (Lutz et al. 1994). In another study, amylin was injected i.p. at a dose of 1μg/kg at dark onset which resulted in a significantly reduced food intake in the first 30 to 60 min after

injection (Lutz et al. 1995). The latter study also showed that the anorectic action of peripheral exogenous amylin seems to be mainly due to a decrease in meal-size especially in the first post-deprivation meal (Lutz et al. 1995; Lutz et al. 2001).

A conditioned taste aversion test is one possibility to find out if a reduction in food intake is due to nausea after administration of a particular substance. Several studies have shwon that amylin does not produce a conditioned taste aversion. Neither an i.p. injection of amylin nor an intrahypothalamic administration of amylin (1 µg), which is a much higher dose than that used in all other previously described experiments with central administration, resulted in a conditioned taste aversion (Chance et al. 1992a; Lutz et al. 1995; Morley et al. 1997). Studies with amylin antagonists confirmed amylin's specific effect on food intake. It was demonstrated that a simultaneous injection of amylin with its antagonists calcitonin gene-related peptide-(8-37) or AC253 abolished the anorectic effect of amylin (Lutz et al. 1996; Watkins et al. 1996). A single peripheral or central injection of the amylin antagonist AC187 increased food intake presumably by neutralizing the effect of endogenous amylin (Mollet et al. 2004; Reidelberger et al. 2004). It therefore can be concluded, that the satiating effect of amylin is specific and that endogenous amylin is of physiological significance for the control of food intake.

2.2.3 Amylin and area postrema (AP)

Various studies demonstrated that the area postrema/nucleus of the solitary tract (AP/NTS) region plays an important role in the control of food intake. First, it receives peripheral satiation signals via splanchnic and vagal afferents from the gastro-intestinal tract. Second, the AP lacks a functional blood brain barrier, so that blood borne signals can directly be monitored in the AP. Furthermore receptors for

anorectic peptides such as amylin, CGRP or CCK have been found in this area. Finally, thermal ablation of the AP resulted in a significant reduction of the anorectic effects of i.p. injected amylin (5 μg/kg), CGRP (5 μg/kg) or sCT (Lutz et al. 1998; Lutz et al 2001).

Immunohistochemical studies for the detection of the expression of the immediate early gene product c-Fos protein, a marker of neuronal activation, showed that the amylin-induced c-Fos response in AP neurons can be effectively blocked by the amylin antagonist AC187. Similar to that of exogenously applied amylin, refeeding, i.e. 2h-access to food following a 24h-food deprivation period, also caused substantial neuronal activation in the AP and NTS whereas fasted control animals showed only minor c-Fos expression. The refeeding-induced c-Fos activation in the AP is presumably caused in part by the release of endogenous amylin because the blockade of amylin binding sites with AC187 significantly reduced the refeeding induced c-Fos expression in AP neurons of rats (Riediger et al. 2004). Peripheral amylin administration also activates c-Fos in the lateral parabrachial nucleus (lPBN), the bed nucleus of the stria terminalis (BNST) and the central nucleus of the amygdala (CeA) (Rowland et al. 1997; Riediger et al. 2004). In AP-lesioned rats, the amylin induced c-Fos expression in the NTS, lPBN and CeA was clearly reduced (Rowland et al. 1999; Riediger et al. 2004). Therefore it can be concluded that the AP is an important primary target area for amylin that is connected to subsequent brain regions.

Electrophysiological in vitro studies showed a direct stimulating effect of amylin on AP neurons, underlining their sensitivity to amylin. Amylin dose dependently stimulated AP neurons with a threshold concentration in the range of circulating plasma amylin concentrations. This finding is consistent with amylin's anorectic effect being dependent on an intact AP. Similar to amylin's effect on food intake, the in vitro

activation of AP neurons by amylin was blocked by AC187 (Riediger et al. 2002; Mollet et al. 2004).

All in all, these findings are consistent with the idea that feeding-induced amylin release activates AP neurons which project to subsequent relay stations known to transmit meal-related signals to the forebrain.

2.2.4 Amylin's long term effect on energy balance

To achieve long-term effectiveness, amylin has to be administered chronically because it has a very short half-life of about 15 min in plasma (Hull et al., 2004). A single amylin injection results in a significant decrease in food intake only in the first hours after administration but a long-term administration of amylin results in a longer lasting inhibition of food intake which may also result in an effect on body weight.

Arnelo et al. (1996) investigated the dose-response effect of peripheral long-term administration of amylin on food intake and meal patterns in rats by using s.c. osmotic minipumps. Amylin decreased food intake and body weight gain dose dependently across the 8-day infusion period. The effect was however transient and most marked on the first days of infusion. The analysis of the effects on meal patterns demonstrated that the anorexia produced by amylin during the dark and light phases occurred through a reduction in the number of meals rather than meal size or meal duration (Arnelo et al. 1996). In contrast, Lutz et al. observed that an i.p. infusion of amylin mainly resulted in a reduction in meal size, rather than in a reduction of the number of meals (Lutz et al. 2001).

A similar meal pattern effect on the inhibition of food intake was observed after central administration. Central infusion of amylin (2.0 pmol/h) via osmotic minipumps over 10 days resulted in a significant decrease of food intake. Consequently, by the

4th day of infusion, amylin-infused rats weighed significantly less than saline controls. This difference persisted throughout the remainder of the infusion period. At sacrifice (day 10), the percent of body weight from retroperitoneal fat depots was significantly lower in the amylin-treated rats, indicative of a reduction in total body adiposity (Rushing et al. 2000). Likewise, Isaksson et al. found a changed lipid metabolism after a chronic amylin treatment via subcutaneous osmotic minipumps (25 pmol/kg/min), which was characterized by decreased adiposity, hypolipidemia and hypoleptinemia but unchanged glucose and protein homeostasis (Isaksson et al. 2005).

The importance of amylin in the control of energy balance over a prolonged time period is also shown in animals with defective leptin or insulin signaling system (e.g. ob/ob mice, db/db mice, Zucker fa/fa rats). Even though the animals are able to keep their body weight at a fairly constant level, this level is markedly above normal (Grabler and Lutz 2004). Food intake and body weight in obese Zucker fa/fa rats, e.g., are increased due to a mutation in the leptin receptor gene, leading to inactivity of this important long-term control system for food intake and body weight (Schwartz 2000). Due to the prevailing insulin resistance in these animals, the animals are hyperinsulinemic and as a consequence of the co-secretion of insulin and amylin also hyperamylinemic. After a chronic infusion with the amylin receptor blocker AC187 Zucker fa/fa rats showed an increase in food intake. This was not observed in the lean control group (Grabler and Lutz 2004). This may indicate that amylin partly takes over the role of the long-term controls of food intake leptin and insulin under certain conditions, especially when the leptin and insulin signaling systems are deficient.

Taken together, these findings demonstrate that centrally or peripherally administered amylin influences food intake and that amylin may play an important role in regulating energy homeostasis.

2.3 Salmon calcitonin (sCT)

The teleost salmon calcitonin (sCT), which has structural similarity to amylin, has been shown to act effectively as an anorectic compound via amylin receptors. Such evidence for the interaction of sCT with amylin binding sites is the partly blocked anorectic action of sCT after CGRP 8-37 or AC187 administration, both antagonists of amylin (Lutz et al. 2000). An intracerebral injection of sCT is more potent than subcutaneous injection, which also indicates that the anorectic action seems to be centrally mediated (Freed et al. 1979). Interestingly, the effect of sCT on food intake is more potent than that of mammalian CT in rats (Twery et al. 1982).

In the subfornical organ, where calcitonin and amylin exert their actions as dipsogens, neuronal excitation by sCT was longer lasting than excitation by calcitonin and amylin. This is in line with the observation that the anorectic action of sCT is prolonged in comparison to that of amylin (Riediger et al. 1999; Riediger et al. 2000; Lutz et al. 2000). This may reflect the apparently irreversible binding of sCT to amylin and CT binding sites, which has been observed in most in vitro test systems (Houssami et al. 1994; Riediger et al. 1999).

2.4 Hormonal influence on energy expenditure

Energy balance depends on energy intake and energy expenditure. Interestingly, besides amylin's well-known effect on energy intake, some studies provided evidence that amylin may also affect the other aspect of energy balance by increasing energy expenditure. Therefore, and to understand how body weight is controlled by amylin, the knowledge of both aspects of energy balance is important.

A recent publication showed that after a 3 week infusion of amylin via subcutaneous osmotic minipumps, energy expenditure was elevated compared to saline-treated

pair-fed and control animals (Roth et al. 2006). Further, Lutz (2006) showed that rats which were food deprived for two days, lost more body weight when treated daily with sCT than fasted control rats. Because in this experiment rats did not have access to food, the data may suggest that sCT stimulates energy expenditure and thereby reduces body weight more during fasting than in controls.

In another study, amylin deficient mice showed an increase in body weight gain compared to wild type control mice (Gebre-Medhin et al. 1998; Lutz 2006) Interestingly, food intake did not differ between the two groups (unpublished). Together, this may indicate that energy expenditure might have been decreased in these knockout mice.

In general, an effect of amylin on energy expenditure could be due to enhanced physical activity, enhanced metabolic rate or raised body temperature. Body temperature after an injection of amylin into the paraventricular hypothalamus of rats in fact resulted in relative hyperthermia of >1°C (Chance et al. 1992b). In another study, a dose dependent hyperthermia was observed with amylin administered into the third ventricle at doses of 1.25 to 20 μg. The maximal hyperthermic effect of about 1.5°C was reached after 30-60 min. The dose at which hyperthermia was first observed was lower for calcitonin gene-related peptide (CGRP) than for rat amylin, suggesting that the response may have been mediated via CGRP receptors (Bouali et al. 1995). A similar response has also been described for salmon calcitonin (Sellami et al. 1993). The doses used in all these experiments, however, are much higher than doses used in the studies assessing amylin's effect on food intake.

3 Aim and hypothesis

The aim of the present study was to investigate the effect of amylin and sCT on energy expenditure. Based on the evidence of a body-weight loss and an increase in body temperature after an acute or chronic amylin or sCT application, we wanted to prove the hypothesis that amylin and sCT increase energy expenditure. First we investigated the effect of amylin on energy expenditure after a single injection. The next step was to repeat this acute experiment with amylin's long lasting agonist sCT. Thereafter, we investigated the effect of a chronic amylin infusion on energy expenditure. Roth et al. (2006) did a similar study with a chronic amylin infusion, using pair-fed animals as a control group for amylin-treated rats. In our study we used instead the more elaborate paradigm of yoked feeding (see 4.2). This procedure allows not only to perfectly match daily food intake but also to exactly imitate the meal pattern.

Because energy expenditure is dependent on various factors like physical activity, thermic effect of food (amount and composition) and body temperature, we recorded physical activity, body temperature, food and water intake. To observe metabolic change, O_2 consumption and CO_2 production were measured and energy expenditure and the respiratory quotient were calculated.

4 Animals, material and methods

4.1 Animals and housing

All experiments were performed with male Wistar rats (Elevage Janvier, Le Genest–St-Isle, France) with a body weight of 300 - 350g at the beginning of the study. All animals were housed individually in metabolic cages (see 4.3) on a layer of wood shavings, in a room with an artificial 12:12-h dark-light cycle (Exp. 1 light on 06.00; Exp. 2- 4 light on 03.00; Exp. 5 and 6 light on 21.00). Rats were adapted to the housing conditions and to handling for at least 1 week before the start of the experiments. All experiments were approved by the Veterinary Office of the Canton Zurich, Switzerland.

4.1.1 Diet

4.1.1.1 Powder chow

All animals had ad libitum access to water and to standard laboratory rat powder chow (3433-9.24 Provimi Kliba, Kaiseraugst), unless otherwise stated (Table 1).

Table 1: Food composition

(Kliba Mühlen, Art. Nr. 3433-9.24, Kaiseraugst, Schweiz)

Dry substance	88.0 %
Protein	18.5 %
Fibre	4.5 %
Fat	4.5 %

Ash	6.3 %
NFE	54.2 %
Energy	12.5 MJ/kg
Starch	35 %

4.1.1.2 Pelleted chow

The 45 mg pellets (Grain-Based Rodent Tablet 1811156, TestDiet®, Richmond USA) were used for the yoked feeding system in experiments 5 (see 4.8.5) and 6 (see 4.8.6) (Table 2).

Table 2: food composition

(Grain-Based Rodent Tablet 1811156, TestDiet®, Richmond USA)

Dry substance	90.9%
Protein	19.9 %
Fibre	4.4 %
Fat	4.7 %
Ash	7.8 %
NFE	54.1 %
Energy	13.8 MJ/kg
Starch	32.8 %

4.2 Yoked feeding

In experiments 5 (see 4.8.5) and 6 (see 4.8.6), we investigated the effect of chronic amylin administration on energy expenditure. In these experiments a saline-infused yoked fed rat was used as control for each amylin treated rat. The reason for this paradigm was, that an amylin injection reduces food intake and this per se results in a decrease of energy expenditure. To account for this confounding factor, a saline-treated rat received a pellet at the same time-point as an amylin-treated rat. This procedure allowed not only a perfect match of daily food intake (as in a normal pair-feeding paradigm) but also to exactly mimic the meal pattern of an amylin-treated rat in the corresponding yoked-fed control. Hence, this allowed us to compare the energy expenditure between the amylin group and the saline group (=yoked fed group), without any confounding influence caused by a difference in total food intake or meal pattern.

Metabolic cages (4.3) were adapted for special food dispensers (Med Associates Inc. St.Albans, VT, USA) of 45 mg pellets (see 4.1.1.2). Entry into the food hopper of an amylin-treated animal interrupted a light beam. Two consecutive light beam breaks triggered the release of a pellet to the food dispenser of the amylin treated rat and simultaneously of the saline-infused yoked fed rat. Hence, both animals received a pellet at exactly the same time point. Delivered pellets were counted by a computer (Software: Med Associates Inc. St.Albans, VT, USA). As a second control group, saline-treated rats were used that had ad libitum access to the food hopper. Pellets were released whenever the rats crossed the light beam of their own food hopper (two light beam breaks/pellet).

4.3 Metabolic cages

An open-circuit indirect calorimetry system was used (Accuscan Instruments, Columbus OH, USA). The 12 Plexiglas cages had a size of 42cm x 42cm x 30cm and were closed with screws to affirm that the cages were air-tight. 6 cages each were connected to one computer, resulting in two independent systems.

4.3.1 Integra System

The integra System contains the following items for different kinds of recordings. All 6 cages were measured in alternating order for 20 seconds each so that all data of each cage were stored every 2nd minute (experiments 5 and 6 every 3rd minute).

4.3.1.1 Animal Activity System (VersaMax)

Physical activity was measured via a grid of infrared light beams. 16 x 16 equally spaced beams traversed the animal cage from front to back and an equal number of beams traversed the same cage from left to right. Vertical sensors monitored rearing or jumping activity. The analyzer determined the animal's position 50 times per second and the summary was stored every 2nd minute. Values were recorded in cm.

4.3.1.2 Food and Liquid Consumption System (DietMax)

Each food or liquid reservoir was placed on a sensitive balance. Differences in balance levels were stored and analysed to calculate food intake and water intake.

4.3.1.3 Oxygen Consumption/Carbon Dioxide Production System (PhysioScan)

V_{O2} (ml/kg/min) and V_{CO2} (ml/kg/min) were measured every second. Based on these measurements energy expenditure and respiratory quotient (RQ = CO_2-production/O_2-consumption) were calculated. Ambient air was pumped through the cage. The air flow was set to 2 litres per minute. The system recorded the average values measured over 20s per cage every 2nd minute in l/min. The system was calibrated with a reference tubing measuring O_2 in the room of 20.94% and a 12h-fasted rat with a respiratory quotient of 0.7 before each experiment.

Energy expenditure was calculated according to Weir (Weir 1949) using the following equation: Total energy expenditure (kcal/h) = (3,9 x ((V_{O2}/1000) x 60)) + (1,1 x ((V_{CO2}/1000) x 60)).

4.3.1.4 Temperature Telemetry System (VersaMon)

The temperature system recorded temperatures from small implantable devices through radio frequency communications. Before implantation of the temperature transmitters they were calibrated by using a jar of water with a defined low and high temperature between 33 and 42°C. Each transmitter had a different calibration curve (temperature versus frequency). The transmitters were implanted in the abdominal cavity with the identity tag in ventral direction (see 4.7.1).

This system was very difficult to calibrate and no measurements were available if the transmitters did not remain in ventral directions, hence whenever the animals moved.

4.3.1.5 Dataquest Telemetry System

Therefore we used a second temperature system in some experiments. The PhysioTel® TA-F40 Small Animal Transmitter (Data Science International Telemetry & Dataquest Software, USA) contained sensors for activity and body temperature and transmitted this information digitally via telemetry to a receiver, which was placed under the cage. Measurements were recorded every 3rd minute. Room temperature was also measured. The transmitters incorporated a magnetically activated “on-off” switch to conserve battery power. This system was used for experiments 5 (see 4.8.5) and 6 (see 4.8.6).

4.4 Peptides

Amylin (Bachem AG; Bubendorf, CH) and salmon calcitonin (Peninsula Laboratories, Inc., USA) were diluted in 0,9% NaCl (Fresenius Kabi AG, Stans, Switzerland). The concentrations used in the different experiments are indicated in 4.8.

4.5 Intraperitoneal (i.p.) injections

For the i.p injections, sterile syringes (Omnifix 1 ml, Braun Melsungen AG, Melsungen, Germany) and sterile needles (Terumo, 26G, Leuven, Belgium) were used. The location of the injection was in the centre of the triangle linea alba – tail onset – thigh onset. The volume of injections was 1 ml/kg.

4.6 Minipumps

For the chronic amylin study (see 4.8.5) osmotic minipumps (Alzet®, mini-osmotic pump, model 2002, Palo Altro, USA) with a mean pumping rate of 0.5 μl/h were implanted (see 4.7.1). The calculated average delivery rate for amylin was 2.0 μg/kg/h during a 14 day period. For the second chronic experiment (see 4.8.6) a pump with a pumping rate of 1 µl/h (Alzet®, mini-osmotic pump, model 2001, Palo Altro, USA) was used. The calculated average delivery rate for amylin was 6 µg/kg/h during an 8 day period. The minipumps were filled with amylin at a concentration of 1,96 mg/ml for experiment 5 and in a concentration of 2.7 mg/ml for experiment 6. The pumps were preincubated in a 0.9% NaCl (Fresenius Kabi AG, Stans, Switzerland) at 37°C for 4h, in order to initiate accurate pumping directly after implantation.

4.7 Surgical procedure

4.7.1 Minipumps and temperature transmitters i.p.

Anaesthesia was induced with 5% isoflurane (IsoFlo®, Provet AG, Lyssach, Switzerland) in a small box. The surgery area was shaved and disinfected (Betadine®, Provet AG, Lyssach, Switzerland). To prevent corneal desiccation an ophthalmic ointment (Vitamin-A Dispersa®, CIBA Vision AG, Niederwangen, Switzerland) was placed on both eyes. The rat was transferred to a nose cone and the anaesthetic level was reduced to 2-3%.

After a 1.5 - 2 cm incision in the skin and the linea alba with scissors, the filled sterile minipumps or temperature transmitters (VM-FA disc, MiniMitter, Bend OR) were placed in the peritoneal cavity. The incision was closed with absorbable suture

material (Vicryl 3-0, Ethicon, Norderstedt, FRG) using a continuous suture for the linea alba and a single bottom suture for the skin.

After surgery rats were injected i.m. with chloramphenicol (0.5 ml/kg, Septicol Suspension 20%, G.Streuli & Co. AG, Uznach, Switzerland) for antibiotic treatment and s.c. with flunixin (0.01 mg/kg; Finadyne®, Provet AG, Lyssach, Switzerland) for analgesic treatment. To avoid postsurgery dehydration a 5 ml depot of 0.9%NaCl (Fresenius Kabi AG, Stans, Switzerland) was injected s.c. The rats were kept in a separate cage with a heat-lamp until they were fully awake. For the following two days rats were treated with chloramphenicol p.o. (Chloropal forte®, Dr. E. Gräub AG, Bern, Switzerland).

4.7.2 Minipumps subcutaneous

The same procedure was used for anesthesia as described under 4.7.1. The minipumps were placed subcutaneously in the neck after a small incision in the skin. The suture was closed using a single bottom suture with absorbable suture material (Vicryl 3-0, Ethicon, Norderstedt, FRG). After surgery rats were treated with antibiotics and analgesics as describe above.

4.7.3 Intraperitoneal cannulas

Anaesthesia was induced with 5% isoflurane (IsoFlo®, Provet AG, Lyssach, Switzerland) and maintained at 2-3% during surgery. The abdomen and the head were shaved and disinfected with Betadine (Betadine®, Provet AG, Lyssach, Switzerland). After a rostro-caudal skin incision on the head, the skin was prepared

sideward to expose the dorsal skull and to allow placement of 4 screws (0.80 x 1/16; Plastics One, Roanoke, VA).

The abdominal incision went through the skin and the muscles beside the linea alba. Thereafter the silicon tubing (O.D. 0.95mm, I.D. 0.5mm; Rubber BV, Hilversum, The Netherlands) was drawn under the skin to the rat's head and connected to an elbow (metal part of 20 gauge needles; B.Braun Melsungen AG, Germany) that was fixed on the scull with dental cement (Technovit 4071; Powder and Solvent, H. Kulzer & Co, GmbH, Wehrheim/Ts., Germany) between the screws. A ring of silicon-glue at the abdominal end of the silicon tubing assured a fixation in the abdominal cavity. Subsequently the abdominal incision was closed with absorbable suture material (Vicryl 3-0, Ethicon, Norderstedt, FRG) using a continuous suture for the muscles and a single bottom suture for the skin. Finally the cannula was flushed with 0.9% NaCl (Fresenius Kabi AG, Stans, Switzerland).

4.8 Experimental design

4.8.1 Experiment 1: acute effect of amylin in ad libitum fed rats

The effect of amylin on energy expenditure was measured using a randomized cross-over design with at least 2 days between trials. Twenty minutes before dark onset, the cages were briefly opened, food was removed and the rats were injected i.p. with 5 μg/kg amylin or saline as control. The rats were returned to the cages, that were closed immediately. Ten minutes later the measurement was started. All parameters were measured during the subsequent 24 hours.

4.8.2 Experiment 2: acute effect of amylin in food restricted rats

The access to the food hopper was blocked from 30 minutes before to 3 hours after injection. 20 minutes before the middle of the light phase, the cages were briefly opened and amylin (1, 5 or 10 μg/kg) or saline as control was injected i.p. The rats were returned to the cages, that were closed immediately. 10 minutes later the measurement was started in the middle of the light phase. Food access was given 3 hours after injection. All parameters were measured during 24 hours, i.e. for 3 hours during food restriction and for the subsequent 21 hours during ad libitum food availability.

4.8.3 Experiment 3: acute effect of amylin administered via i.p. catheter in food restricted rats

One hour before the start of the experiment a clear polyethylene tubing (O.D. 1.45mm, I.D. 0.5mm; PE 50, Rubber BV, Hilversum, the Netherlands) filled with saline was connected from the top of the cage through a small hole in the cage to the metal elbow on the head of each animal for the i.p. injections. Thereafter the cages were closed and the rats were fasted. 30 minutes later, rats were injected i.p. through the cannula with amylin (1, 5 or 10 μg/kg). The tubing was flushed with 0.2 ml saline. An air bubble between amylin and saline assured visual control that all amylin was injected. Rats were injected in a randomized cross-over design with a minimal interval of two days between trials.

4.8.4 Experiment 4: acute effect of salmon calcitonin in food restricted rats

In this experiment with a similar design as in experiment 2, energy expenditure was measured after an injection with the amylin agonist salmon calcitonin (0.1, 1 or 5 μg/kg) or saline as control. The trials were separated by at least three days because of sCT's long lasting effect on food intake (Lutz et al. 2000).

4.8.5 Experiment 5: chronic effect of amylin (2 µg/kg/h i.p.) in rats

After the implantation of the minipumps and the temperature transmitters (see 4.7.1), the measurements were recorded over 10 days. The experiment was performed in 2 trials using 2 groups of animals because of the capacity of our Accuscan system. Every day between 8.00 and 9.00 (light on: 21.00 – 9.00) the systems were stopped, data collected and the cages were opened to weigh the rats. Cages were cleaned twice per week during the same time window. Food and water were refilled and the systems were restarted. Due to amylin's inhibiting effect on food intake, which already results in a decrease in energy expenditure, we used a yoked saline-treated animal as a second control for each amylin-treated animal (see 4.2).

4.8.6 Experiment 6: chronic effect of amylin (6 µg/kg/h s.c.) in rats

Temperature transmitters were implanted (see 4.7.1) one week before the start of the experiment to avoid a body weight loss in the first days caused by the surgery. Just before the start of the experiment minipumps were implanted subcutaneously in the neck (see 4.7.2). The measurements were recorded over 7 days. The experiment was repeated once with a new group of animals. Every day between 8.00 and 9.00

the systems were stopped, data collected and the cages were opened to weigh the rats and clean the cages. Food and water were refilled and the systems were restarted. Due to amylin's inhibiting effect on food intake, we used a yoked-fed group of saline-infused animals as a second control group as in experiment 5 (4.2 and 4.8.5).

4.9 Statistical analysis

The data are expressed as mean ± SE. One-way ANOVA with repeated measures and post-hoc Bonferroni test for each time point were used to test for significant differences, unless otherwise stated. $P<0.05$ was considered significant.

5 Experiments and results

5.1 Experiment 1: acute effect of amylin in ad libitum fed rats

In animals with ad libitum access to food, amylin (5 μg/kg) had no effect on energy expenditure compared to control when injected just before dark onset (Figure 1). Food intake was significantly reduced compared to controls 30 minutes, 1 hour ($p<0.01$) and 2 hours ($p<0.05$) after injection (Figure 2). Water intake was decreased to a similar degree as food intake (Figure 3). RQ (Figure 4), physical activity and body temperature (data not shown) were not different from control.

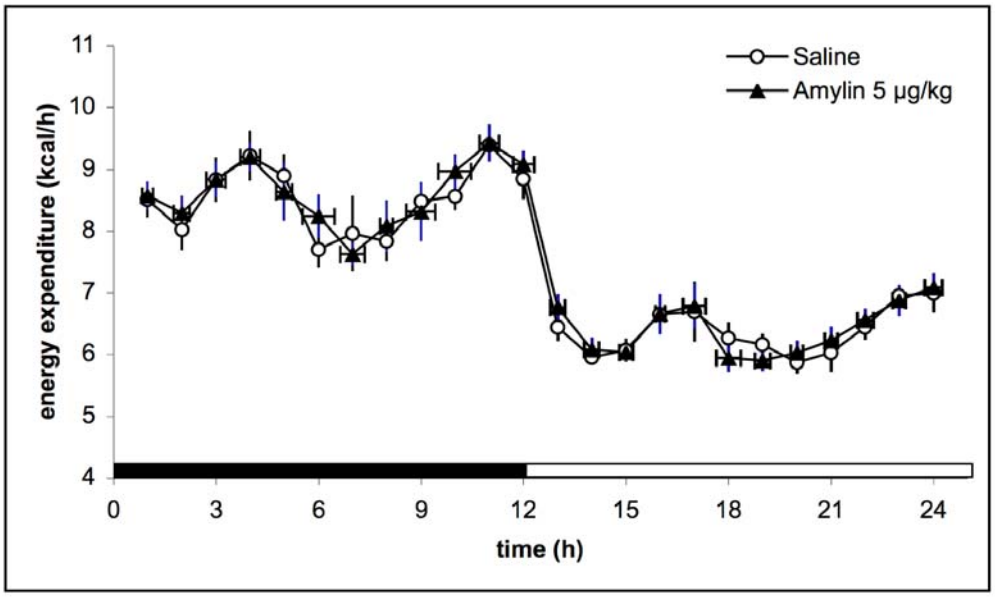

Figure 1: Effect of amylin (5 μg/kg i.p.) or saline on energy expenditure. Rats (n = 10) were injected in a randomized cross-over design just before dark onset. Data are expressed as mean ± SE for every hour.

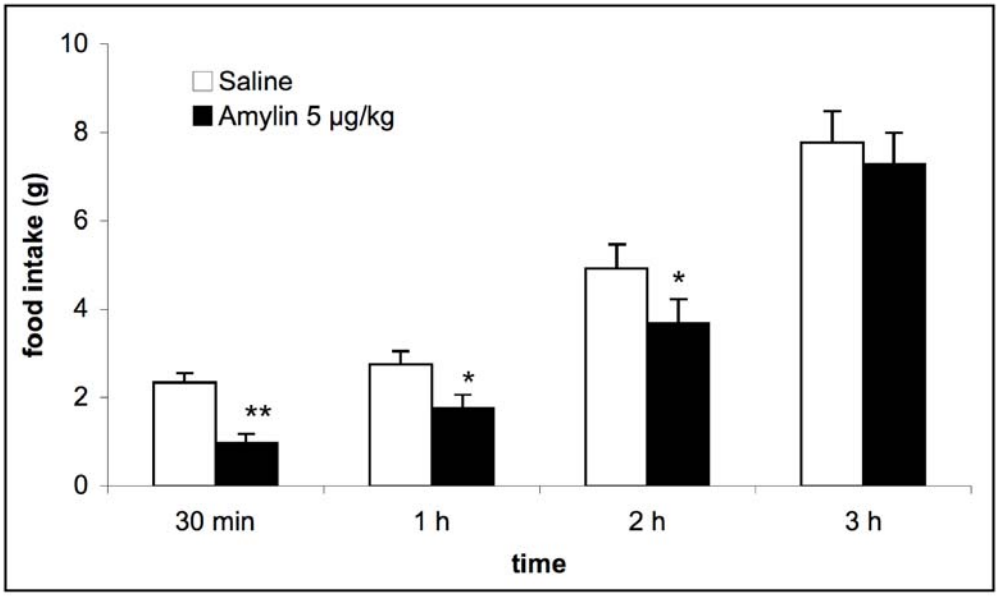

Figure 2: Effect of amylin (5 μg/kg i.p.) or saline on cumulative food intake over the first 3h after injection. Rats (n = 10) were injected in a randomized cross-over design just before dark onset. Data are expressed as mean ± SE. *p<0.05, **p<0.01 (paired t-test).

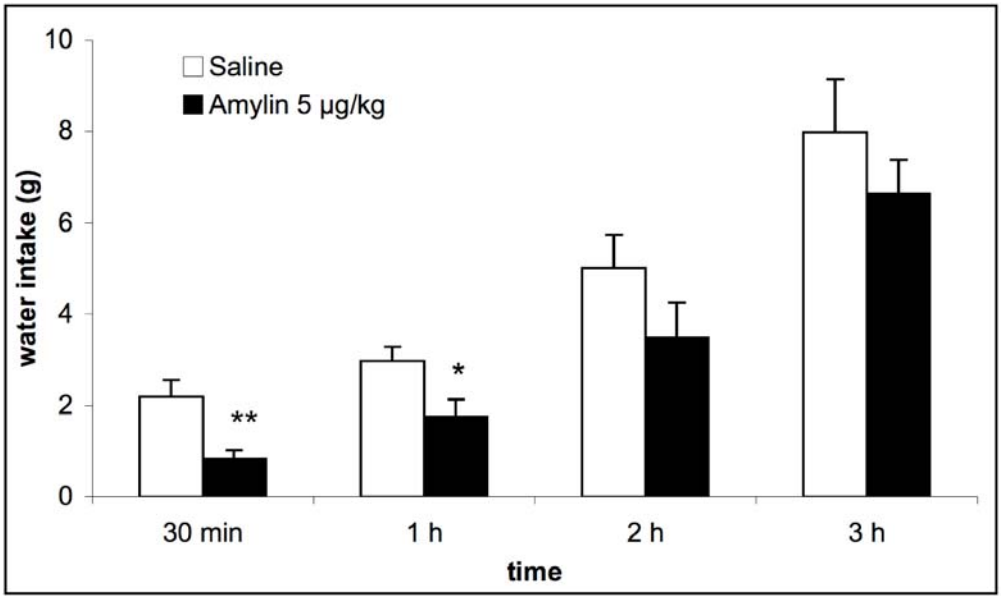

Figure 3: Effect of amylin (5 μg/kg i.p.) or saline on cumulative water intake over the first 3h after injection. Rats (n = 10) were injected in a randomized cross-over design before dark onset. Data are expressed as mean ± SE. *p<0.05, **p<0.01 (paired t-test).

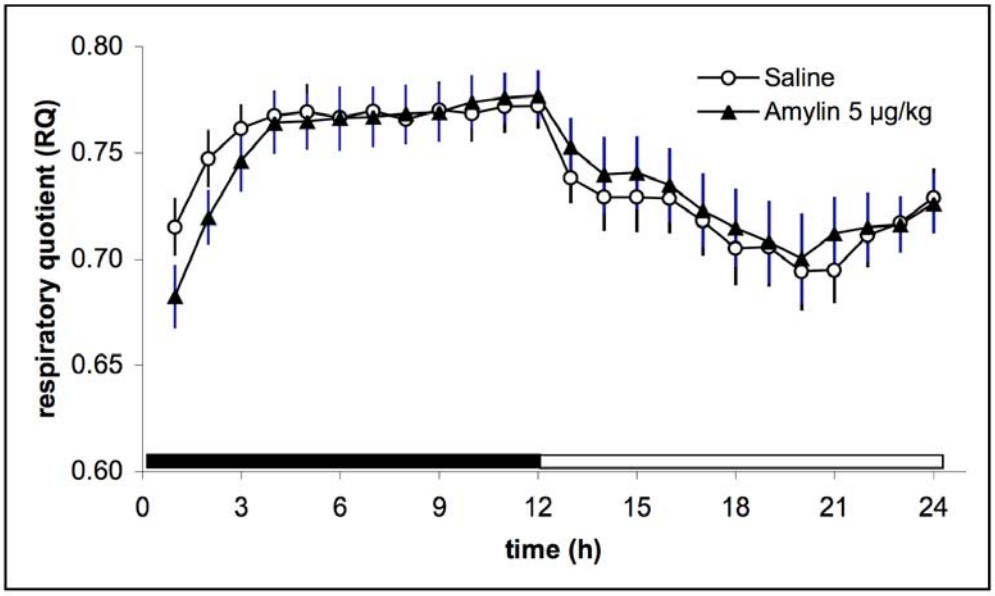

Figure 4: Effect of amylin (5 μg/kg i.p.) or saline on respiratory quotient (RQ). Rats (n = 10) were injected in a randomized cross-over design just before dark onset. Data are expressed as mean ± SE.

5.2 Experiment 2: acute effect of amylin in food restricted rats

In this experiment, amylin was injected in the middle of the light phase at 3 different doses (1, 5 and 10 μg/kg) in rats without access to food. Energy expenditure was slightly but not significantly increased in the first 30 minutes. Overall no effect of amylin on energy expenditure was observed (Figure 5). Physical activity, respiratory quotient and body temperature (data not shown) were not different from control. After refeeding, i.e. 3 hours after injection, no difference in any of the measured parameters was observed (data not shown).

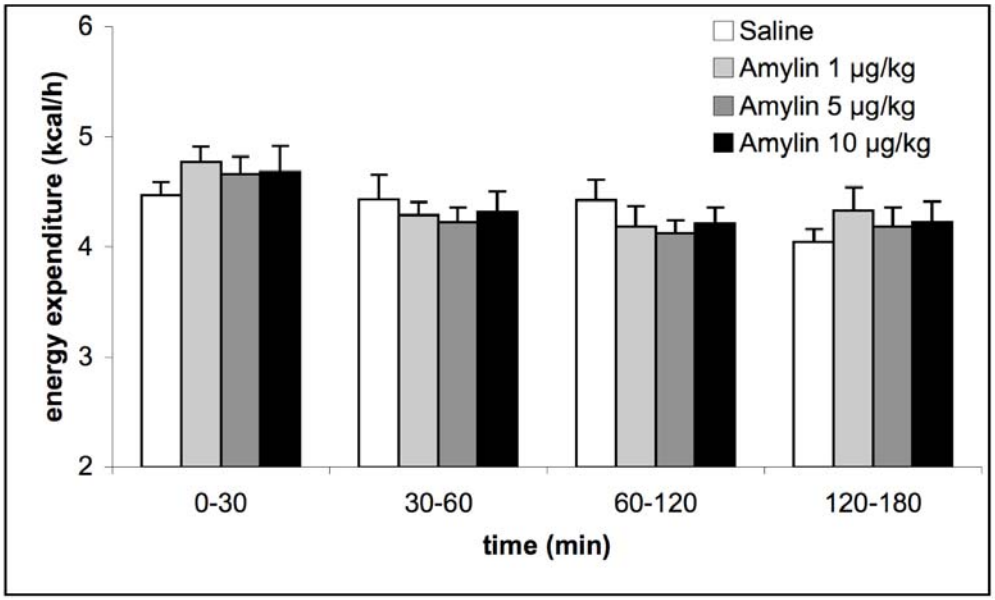

Figure 5: Effect of amylin (1, 5 or 10 µg/kg i.p.) on energy expenditure. Rats (n = 10) were injected in the middle of the light phase in a randomized cross-over design. Data are expressed as mean ± SE.

5.3 Experiment 3: acute effect of amylin administered via i.p. catheter in food restricted rats

One technical problem in the previous experiments was that the airtight metabolic cages had to be opened briefly for the administration of amylin. After that, it took some time for a stable O_2/CO_2 measurement to be reached again. It may well be that the equilibration of the atmosphere in the metabolic cages after injecting the animals might have taken too long for the recording system to detect an effect of amylin on energy expenditure. To circumvent this problem cages were closed at least 30min before the start of the drug administration in experiment 3 and rats were injected through an i.p. canula (see 4.7.3). An unexpected problem was that rats often bit the tubing within the first 3h of recordings. This resulted in a higher physical activity, so

that every animal which bit the tubing had to be excluded from the experiment (in total four rats). Two rats had to be excluded because the tubing was leaking or blocked.

After acute i.p. amylin infusion in food-restricted rats in the middle of the light phase, there was no difference in energy expenditure compared to control rats in any group (1, 5 and 10 µg/kg) (Figure 6). Furthermore no effect on RQ or physical activity was found (data not shown).

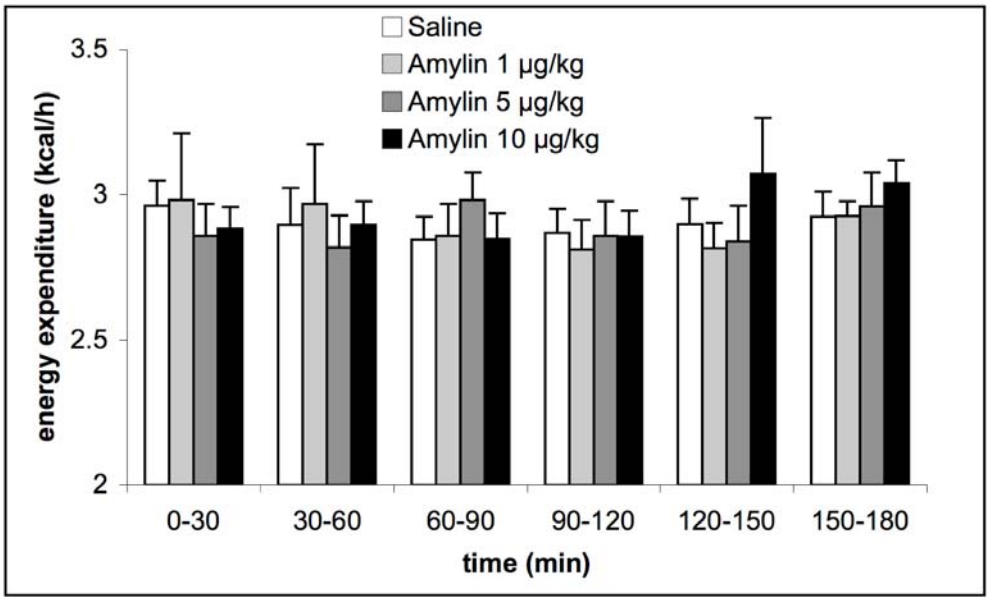

Figure 6: Amylin had no effect on energy expenditure after an acute injection of amylin (1, 5 and 10 µg/kg) via i.p. canulas. Rats (saline n=9; amylin 1 µg/kg n=6; amylin 5 µg/kg n=7; amylin 10 µg/kg n=8) were injected in a randomized cross-over design in the middle of the light phase. Data are expressed as mean ± SE (ANOVA one way).

5.4 Experiment 4: acute effect of salmon calcitonin in food restricted rats

Because amylin tended to increase energy expenditure in experiment 2 (see 5.2), the next step was to use amylin's long acting agonist salmon calcitonin, in a similar design than in experiment 2. SCT was injected in the middle of the light phase at 3 different doses (0.1, 1 and 5 µg/kg) in rats that had no access to food for three hours after injection. Energy expenditure was significantly increased 120 min ($p<0.01$) and 180 min ($p<0.01$) after injection of the highest dose of sCT (5 µg/kg) compared to control (Figures 7 and 8). The 1 µg/kg dose just failed to reach significance compared to control at t=120 min ($p=0.077$) (Figures 7 and 8). RQ was significantly increased by 5 µg/kg sCT at t=120 min ($p<0.01$) (Figure 9).

After refeeding energy expenditure was significantly decreased in rats injected with sCT compared to control (1 µg/kg: t=7h $p<0.05$; normality failed; Dunn's Method; 5 µg/kg: t=8h $p<0.05$ and t=10h $p<0.01$) (Figure 7). Cumulative food intake was significantly reduced at t=6h ($p<0.05$) and t=8h ($p=0.01$) in rats injected with 1 µg/kg (Figure 10). With the dose of 5 µg/kg, food intake was reduced during the entire experiment when food was present, i.e. between t=3h and t=24h (t=24h: $p<0.05$) (Figure 10). Food intake was normalized during the wash-out period, indicating that there was no carry-over between trials.

After refeeding RQ was significantly decreased by sCT (5 µg/kg) from t=4h ($p<0.01$) to t=16h ($p<0.05$) (Figure 10).

Physical activity (Figure 11), body temperature and water intake were not significantly different between the groups at any time point during the food restriction from t=0 to t=3h (data not shown).

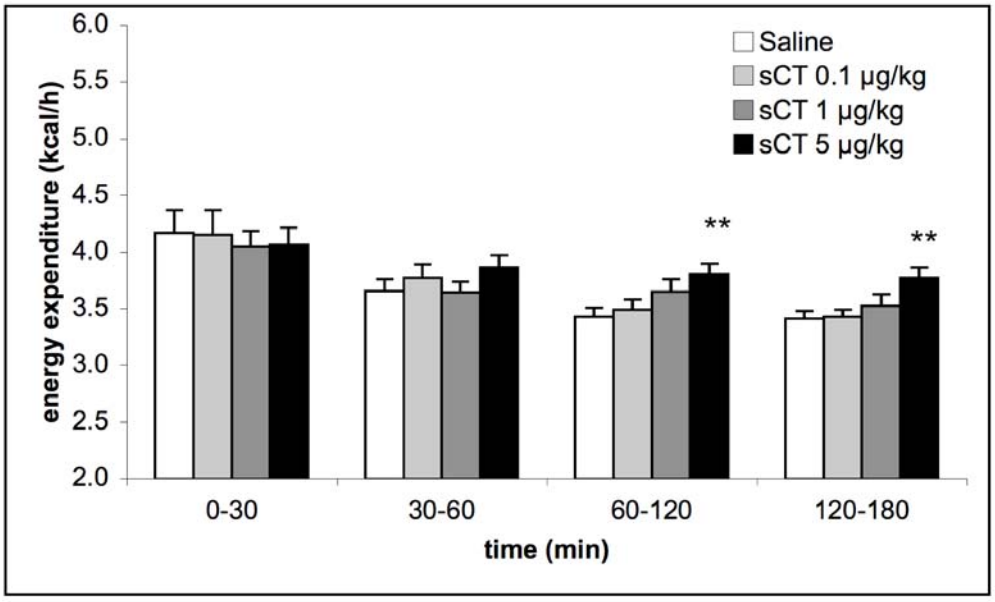

Figure 7: Effect of sCT (0.1 µg/kg, 1 µg/kg or 5 µg/kg i.p.) or saline on energy expenditure in the first 3 h after injection when rats had no access to food. Rats (n = 10) were injected in a randomized cross-over design in the middle of the light phase. Data are expressed as mean ± SE. **p<0.01, sCT 5 µg/kg vs. saline (ANOVA one way).

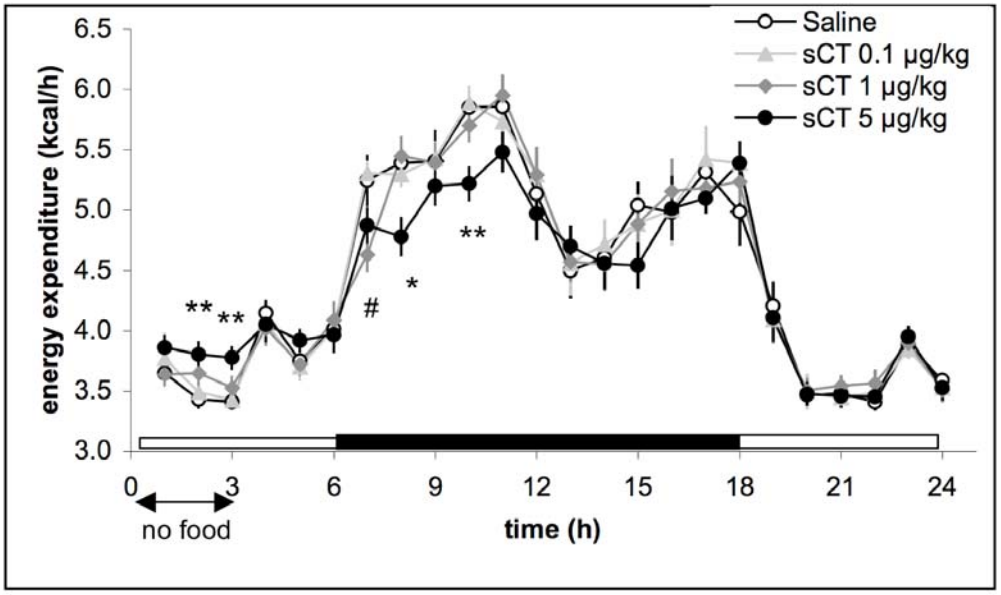

Figure 8: Twenty-four hour time course of energy expenditure after injection of sCT (0.1 µg/kg, 1 µg/kg or 5 µg/kg i.p.) or saline. Rats (n = 10) were injected in a randomized cross-over design in the middle of the light phase. Data are expressed as mean ± SE. #p<0.05 sCT 1 µg/kg vs. saline. *p<0.05, **p<0.01 sCT 5 µg/kg vs. saline (ANOVA one way).

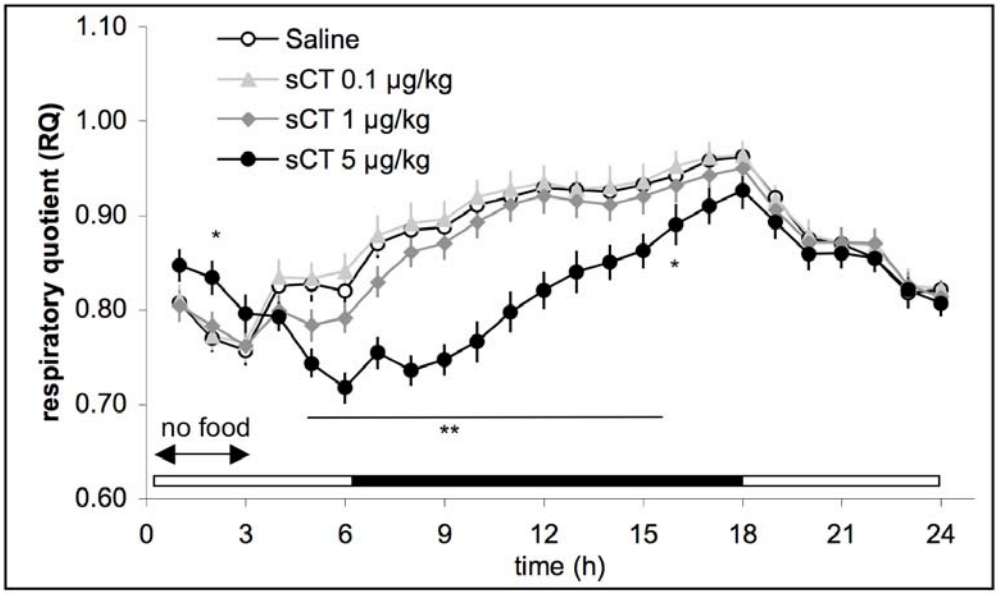

Figure 9: Twenty-four hour time course of the respiratory quotient after injection of sCT (0.1 µg/kg, 1 µg/kg or 5 µg/kg i.p.) or saline. Rats (n = 10) were injected in a randomized cross-over design in the middle of the light phase. Data are expressed as mean ± SE. *p<0.05, **p<0.01 sCT 5 µg/kg vs. saline (ANOVA one way).

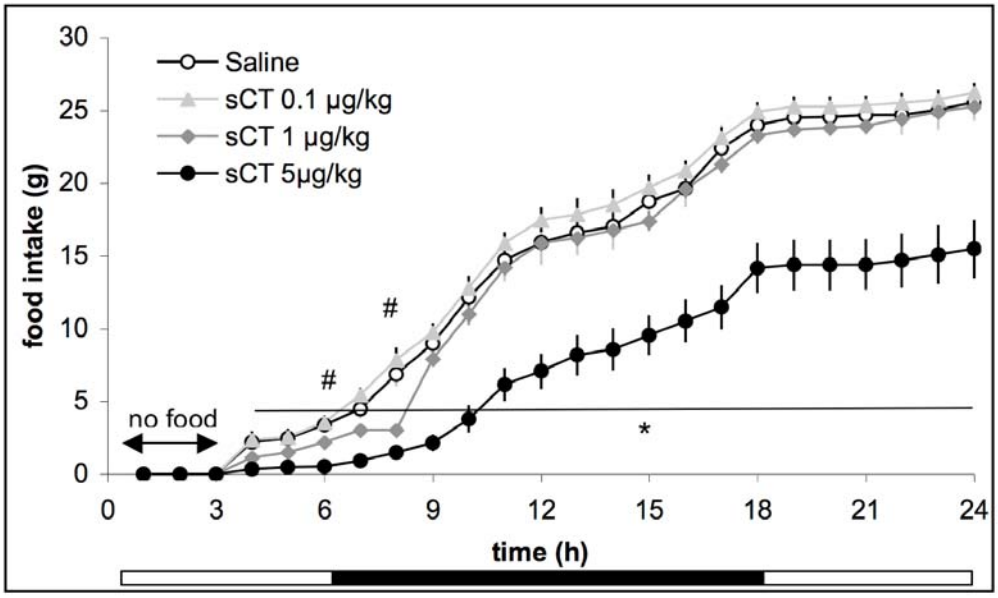

Figure 10: Cumulative food intake after injection of sCT (0.1 µg/kg, 1 µg/kg or 5 µg/kg i.p.) or saline. Rats (n = 10) were injected in a randomized cross-over design in the middle of the light phase. Data are expressed as mean ± SE. $^{\#}p<0.05$ sCT 1 µg/kg vs. saline. *p<0.05, sCT 5 µg/kg vs. saline (ANOVA one way).

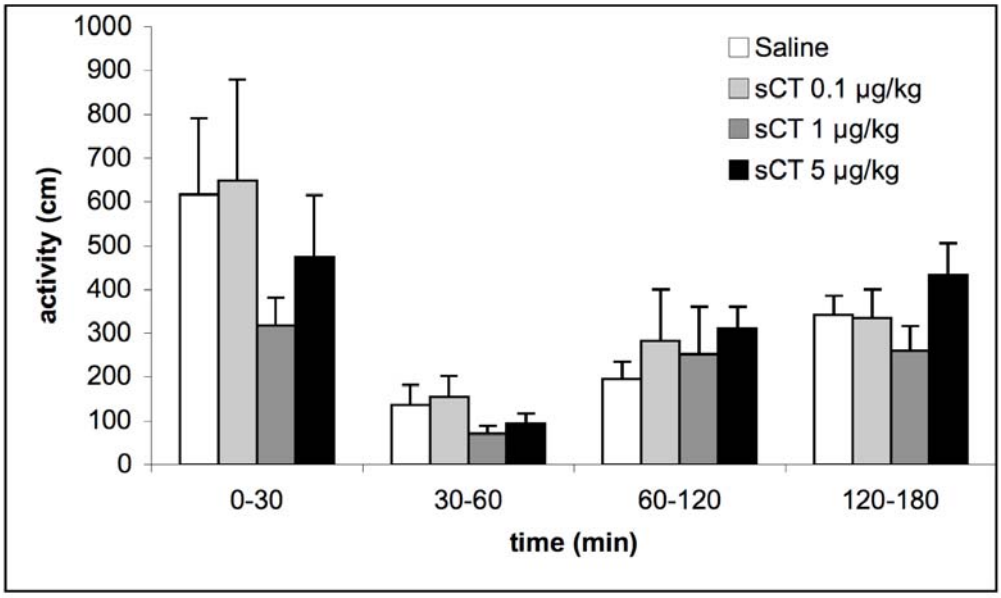

Figure 11: No significant effects were observed on physical activity after an acute injection of sCT (0.1, 1 or 5 µg/kg) in rats (n = 10) in the middle of the light phase. Data are expressed as mean ± SE (ANOVA one way).

5.5 Experiment 5: chronic effect of amylin (2 µg/kg/h i.p.) in rats

In this experiment amylin was infused via i.p. minipumps at a dose of 2 µg/kg/h over 10 days. Saline infusion was used as control. The third group of rats was saline infused but yoked with the amylin-treated group.

Amylin slightly reduced food intake on days 1-3 of the infusion. This effect did not quite reach significance on any particular day (Figures 13 and 17). Figures 12 – 14 show the recordings for energy expenditure, cumulative food intake and body temperature from day 1 over 23 hours. The drop in energy expenditure and body temperature between the light and the dark phase is associated with a circadian pattern of lower activity in the light phase. Due to this difference between dark and

light phase, daily average recordings are calculated and shown separately for dark and light phase. Cumulative food intake on day 1 (Figure 13) was slightly decreased in amylin-treated rats. Food-intake in yoked-fed rats corresponded exactly to the amount of food in the amylin-treated rats. Rats in all groups ate more during the first 12 hours (dark phase). In the light phase of day 1, body temperature appeared to be slightly lower in the yoked fed group compared to the amylin or saline control (Figure 14). For the following days only 12-h dark phase average values and light phase average values (11 h) are shown for most parameters.

The strong reduction of energy expenditure in the dark phase in all groups on day 1 was most likely caused by the surgery on day 0 (Figure 15). Although the effect of amylin on energy expenditure was not significant, it seems that for the following days amylin clearly prevented the decrease in energy expenditure compared to the yoked group in the light phase as well as in the dark phase (Figures 15 and 16). Lower energy expenditure in the yoked fed rats compared to the saline ad libitum fed rats was expected because the reduced food intake in the yoked fed rats physiologically results in a decrease in energy expenditure. In general, energy expenditure in the dark phase was higher compared to the light phase, most likely in part due to higher physical activity in the dark phase. The observed effect of amylin on energy expenditure compared to yoked rats was slightly stronger in the light phase than in the dark phase (Figures 15 and 16).

No change in body weight was observed in the saline group after surgery on day 0. These rats started to gain body weight after day 5. Amylin-treated rats significantly lost body weight from day 1 to 4 compared to the saline group. The difference to the yoked group was only seen on day 1. The amylin group slightly gained body weight after day 3 but tended to have a lower body weight during the entire experiment than the saline group and reached levels of the yoked group after day 5. The yoked-fed

rats did not loose body weight after surgery on day 0. The biggest body weight loss in the yoked-fed group occurred between days 1 and 2. On the following days they slightly gained body weight (Figure 18).

The effect of amylin on body temperature was stronger in the light phase than in the dark phase. Body temperature in the light phase was significantly increased on days 1, 3 and 5 in the amylin group compared to the yoked-fed rats (Figure 20). No effect was observed on physical activity (Figures 21 and 22) and RQ (data not shown).

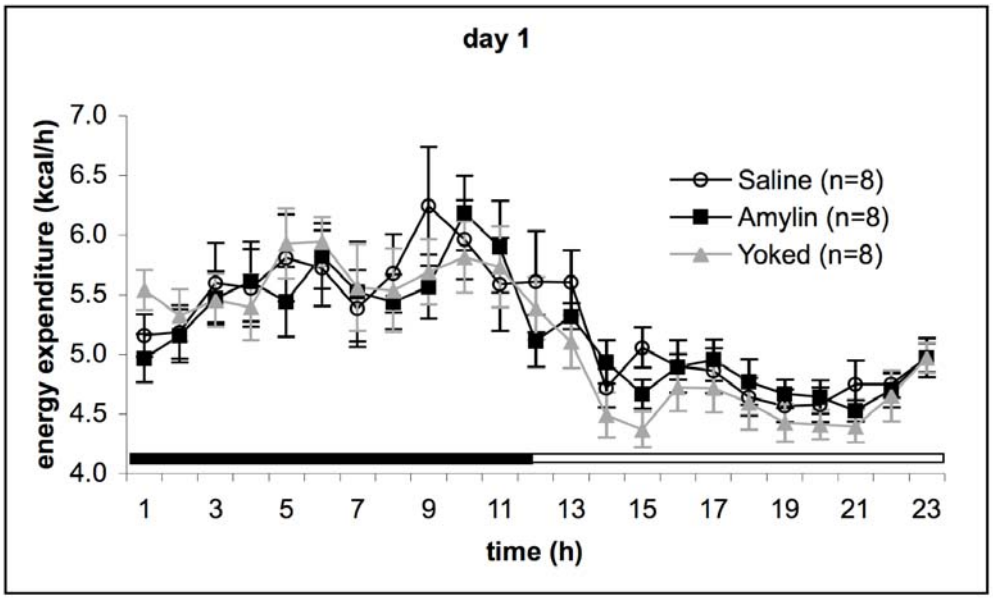

Figure 12: Effect of amylin (2 µg/kg/h i.p.) or saline infusion on energy expenditure on day 1 in rats (n = 8). Data are expressed as mean ± SE for every hour (ANOVA one way).

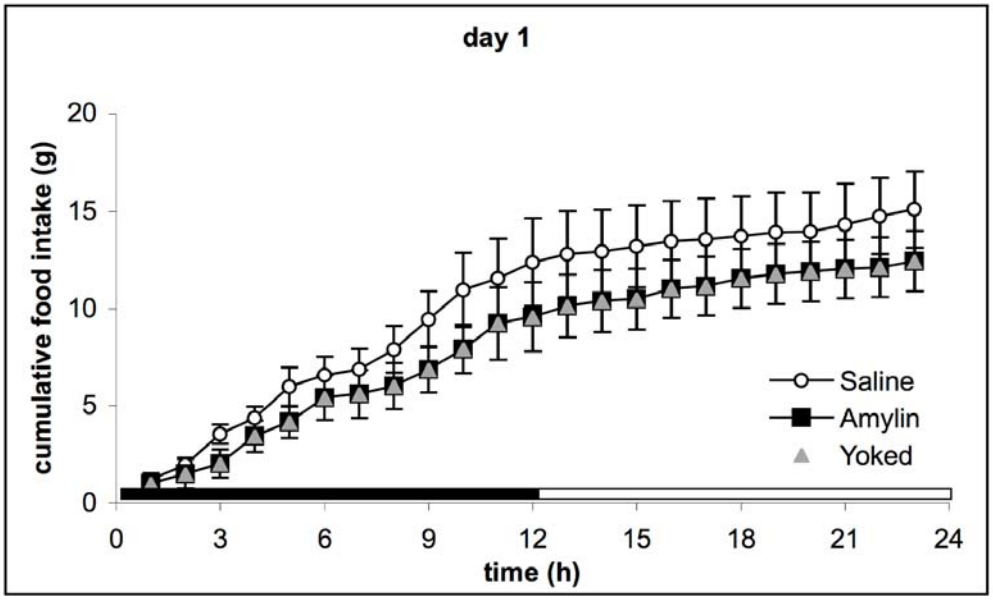

Figure 13: Effect of amylin (2 µg/kg/h i.p.) or saline infusion on cumulative food intake on day 1 in rats (n = 8). Yoked fed rats were infused with saline but their food intake was yoked to that of amylin infused rats. Data are expressed as mean ± SE for every hour (ANOVA one way).

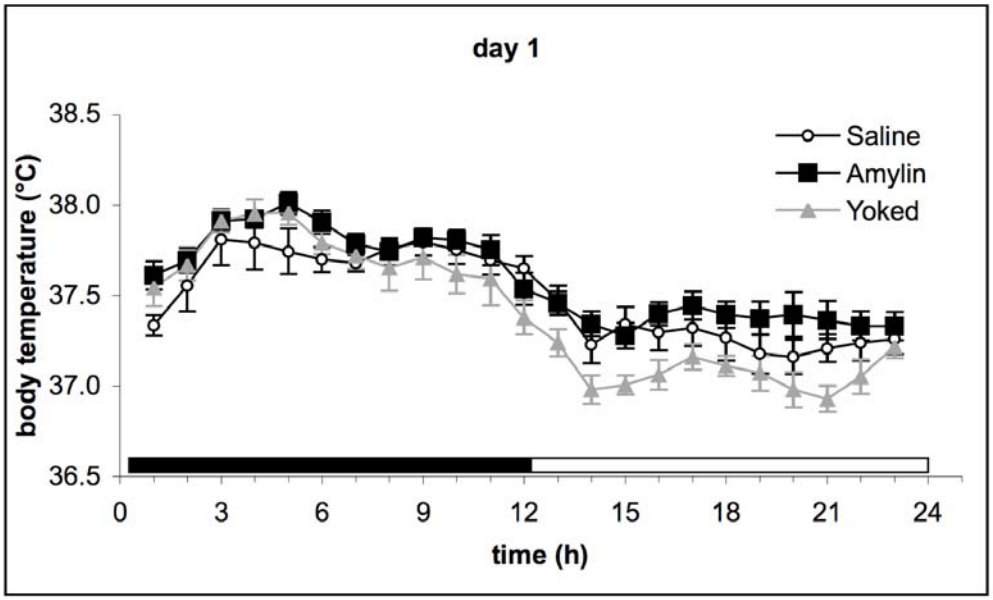

Figure 14: Effect of amylin (2 µg/kg/h i.p.) or saline infusion on body temperature on day 1 in rats (n = 8). Data are expressed as mean ± SE for every hour (ANOVA one way).

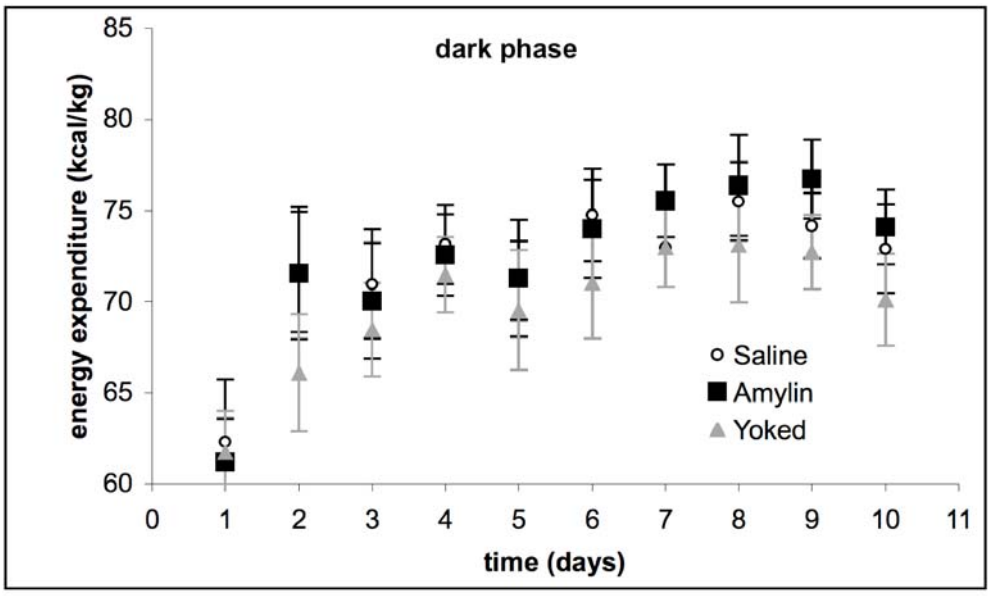

Figure 15: Average dark phase energy expenditure during chronic infusion of amylin (2 µg/kg/h) or saline in rats (n = 8). Data are

expressed as mean ± SE for the dark phase of every day (ANOVA one way).

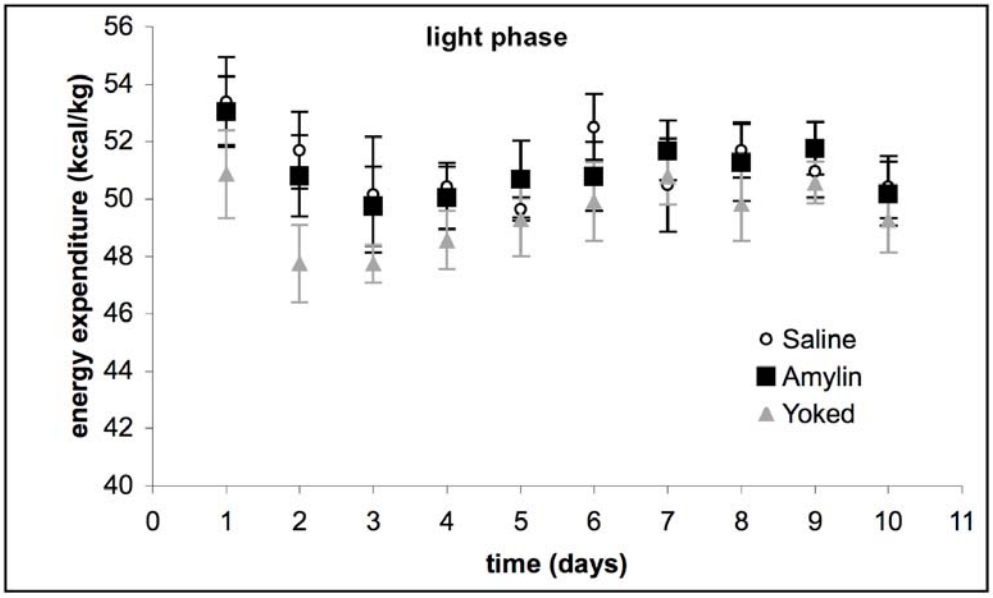

Figure 16: Average light phase energy expenditure during chronic infusion of amylin (2 µg/kg/h) or saline in rats (n = 8). Data are expressed as mean ± SE for every day (ANOVA one way).

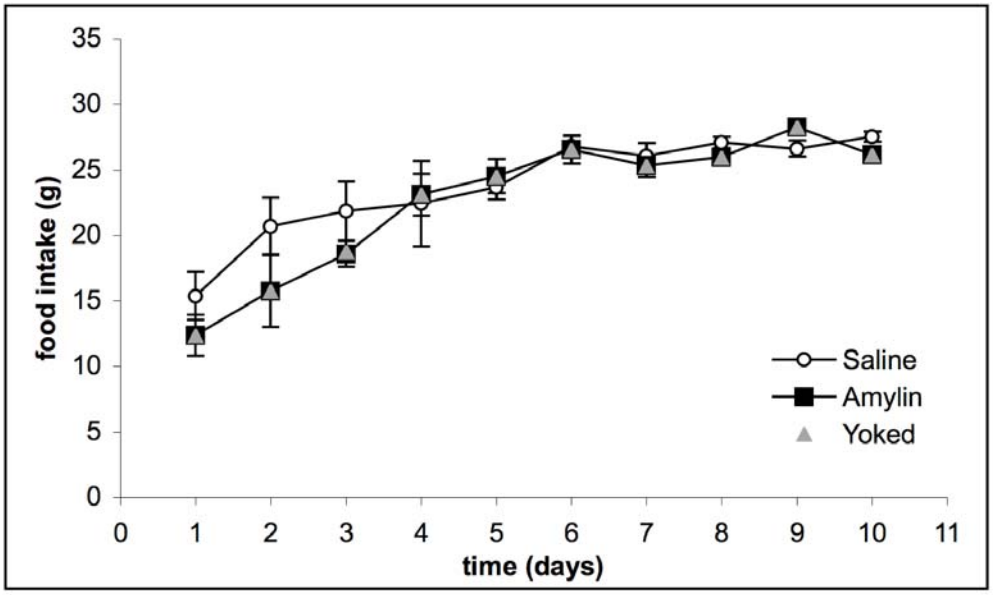

Figure 17: Effect of a chronic amylin infusion (2 µg/kg/h i.p.) on daily food intake in rats (n = 8). Yoked fed rats were infused with saline but their food intake was yoked to that of amylin infused rats. Data of average daily food intake are expressed as mean ± SE (ANOVA one way).

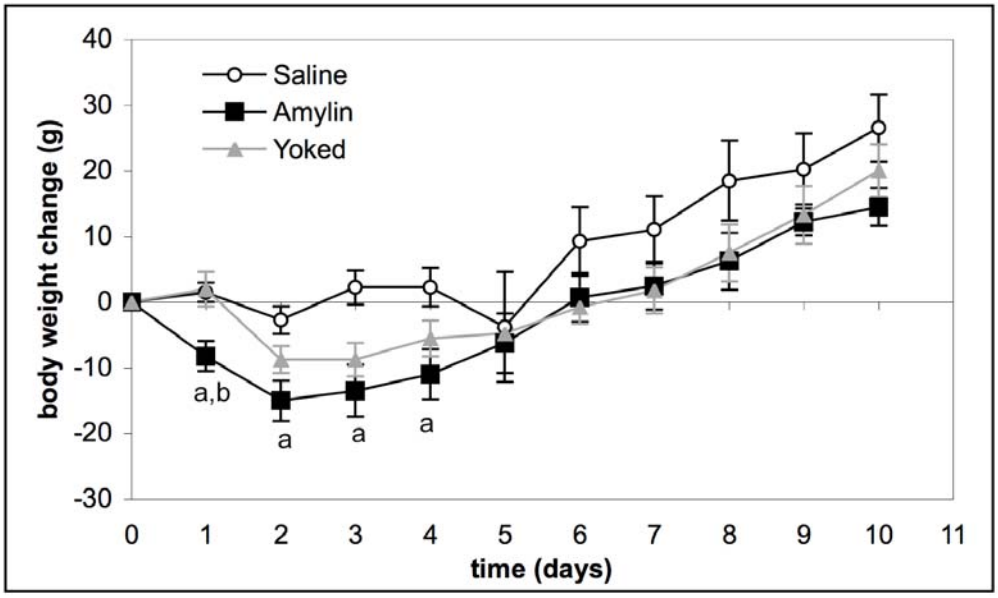

Figure 18: Effect of chronic amylin infusion on body weight change in rats (n = 8). Amylin treated rats lost significantly more body weight on days 1-4 compared to saline. The difference between amylin and yoked rats was significant on day 1. Data are expressed as mean ± SE for every day. a: $p<0.05$ amylin vs. saline; b: $p<0.05$ amylin vs. yoked (ANOVA one way).

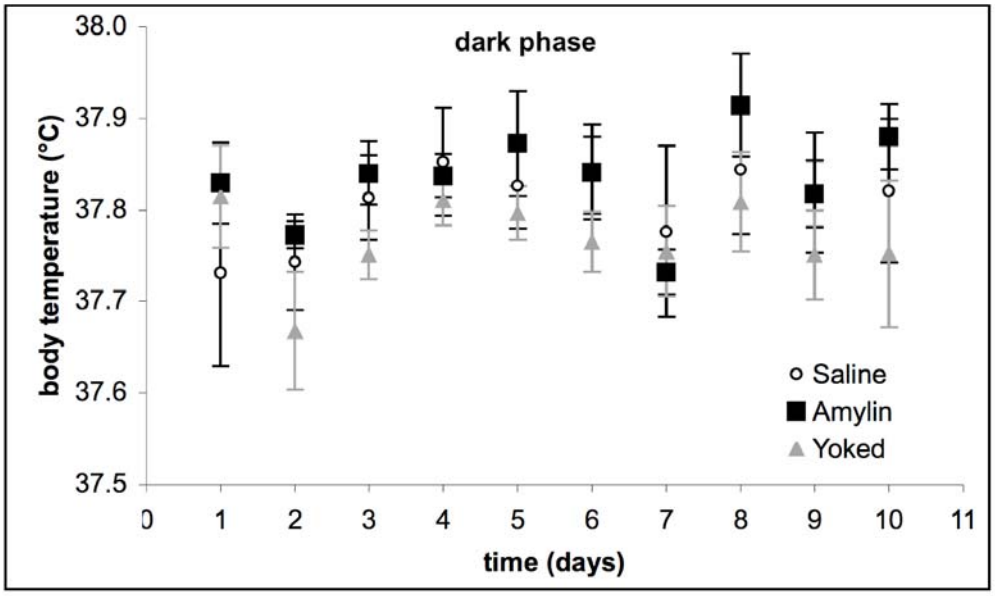

Figure 19: Average dark phase body temperature during chronic infusion of amylin (2 µg/kg/h) or saline in rats (n = 8). Data are expressed as mean ± SE for every day (ANOVA one way).

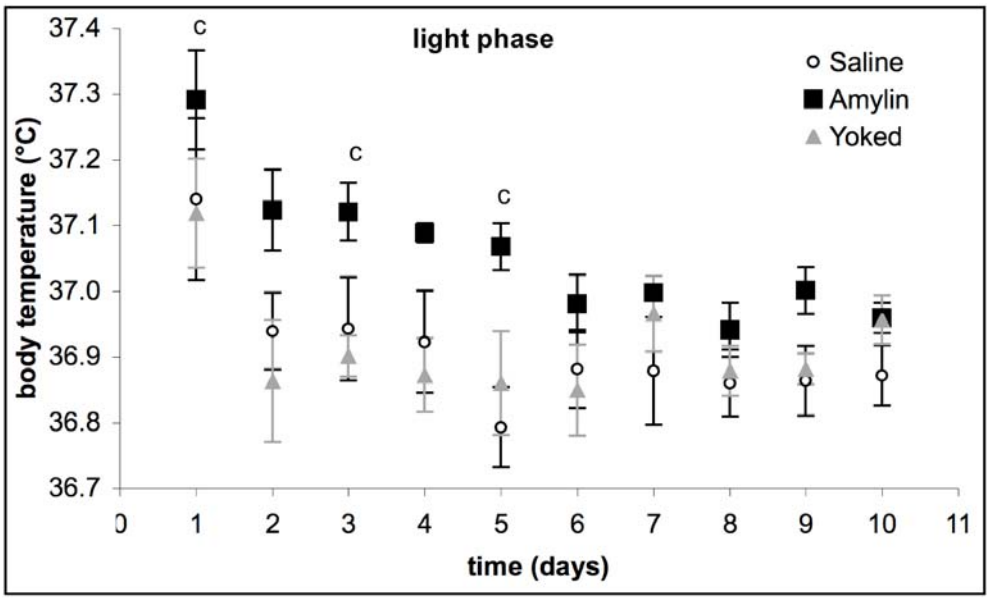

Figure 20: Average light phase body temperature during chronic infusion of amylin (2 µg/kg/h) or saline in rats (n = 8). Data are

expressed as mean ± SE for every day. c: $p<0.05$ amylin vs. yoked (ANOVA one way).

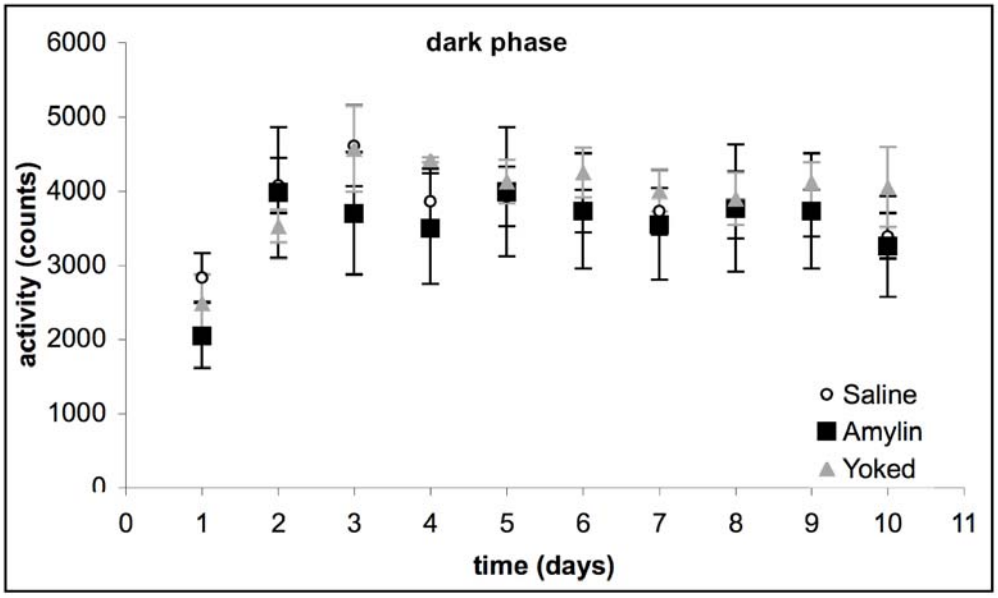

Figure 21: Average dark phase physical activity during chronic infusion of amylin (2 µg/kg/h) or saline in rats (n = 8). Data are expressed as mean ± SE for every day (ANOVA one way).

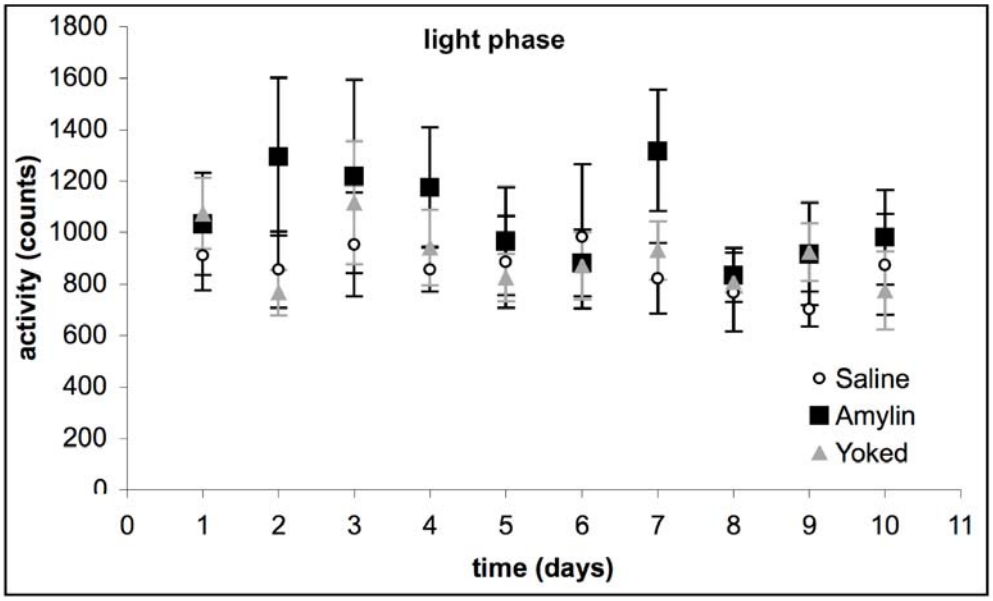

Figure 22: Average light phase physical activity during chronic infusion of amylin (2 µg/kg/h) or saline in rats (n = 8). Data are expressed as mean ± SE for every day (ANOVA one way).

5.6 Experiment 6: chronic effect of amylin (6 µg/kg/h s.c.) in rats

In the second chronic experiment, the dose of amylin was increased to 6 µg/kg/h. Amylin was infused s.c. and data were recorded for 7 days. Energy expenditure was significantly reduced in amylin treated rats and the yoked rats on day 1 in the light and in the dark phase (Figures 23 and 24). This decrease is probably caused by the strong amylin-induced reduction of food intake on day 1 (see Figure 25). Amylin treated rats were not different from the saline group on day 2 although food intake was still significantly reduced. Further, yoked fed animals had a significantly reduced energy expenditure on days 6 and 7 in the dark phase, while amylin treated rats were not different from control. These effects indicate that amylin clearly prevented a

decrease of energy expenditure when associated with the amount of food eaten (Figures 23 and 24).

Food intake in amylin treated animals and in the yoked rats was significantly reduced on days 1 and 2 and regained control levels on the following days (Figure 25).

Saline treated rats did not loose body weight after implantation of the minipumps. The amylin treated group lost body weight on day 1 compared to the saline group. Body weight of amylin treated rats after day 3 was slightly lower for the rest of the treatment period compared to the saline group. Yoked fed rats lost significantly more body weight on days 1 and 2 and they had a significantly lower body weight compared to saline control during the entire experiment (Figure 26).

Body temperature of amylin treated rats was not different from the saline group. Yoked fed rats had a significantly lower body temperature compared to the saline group on days 4, 5 and 7 in the dark phase and on days 1, 3, 6 and 7 in the light phase. During the entire treatment period yoked fed animals had a lower body temperature than amylin treated rats (Figures 27 and 28).

No effect was observed on physical activity (Figures 29 and 30) and RQ (data not shown).

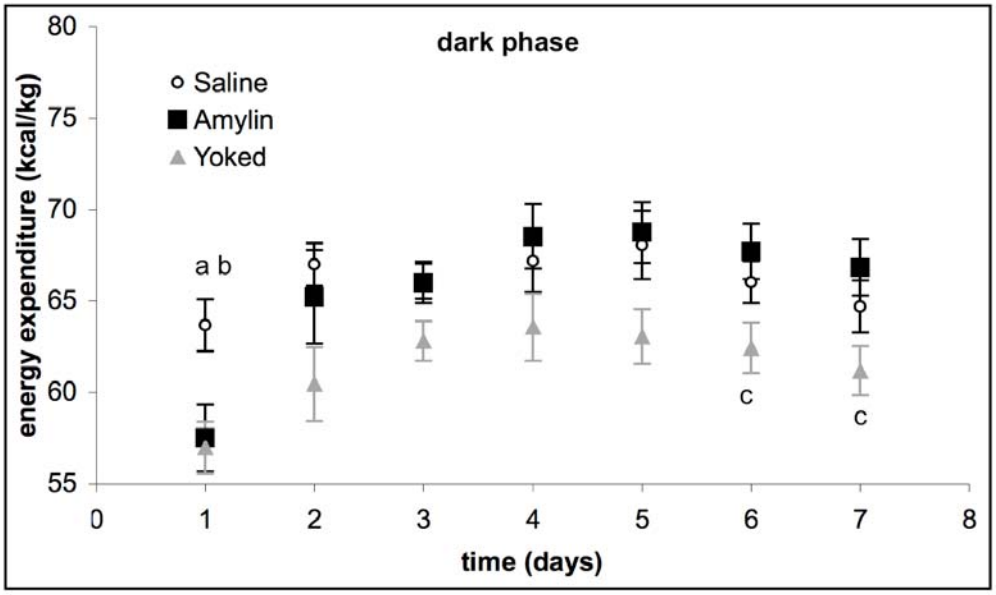

Figure 23: Average dark phase energy expenditure during chronic infusion of amylin (6 µg/kg/h) or saline in rats (n = 8). Data are expressed as mean ± SE for every day. a: $p<0.05$ amylin vs. saline, b: $p<0.05$ yoked vs. saline, c: $p<0.05$ amylin vs. yoked (ANOVA one way).

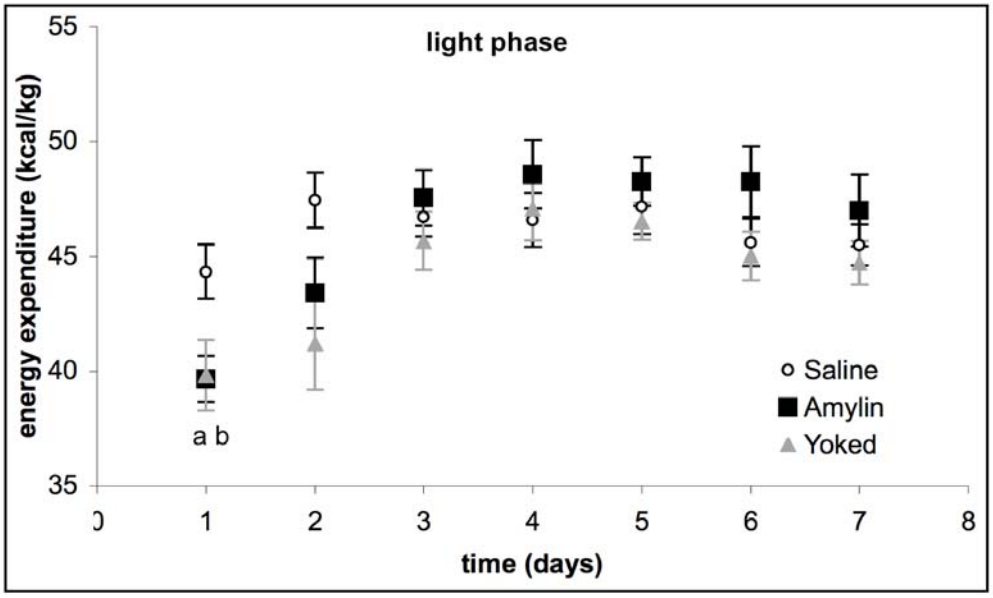

Figure 24: Average light phase energy expenditure during chronic infusion of amylin (6 µg/kg/h) or saline in rats (n = 8). Data are expressed as mean ± SE for every day. a: $p<0.05$ amylin vs. saline; b: $p<0.05$ yoked vs. saline (ANOVA one way).

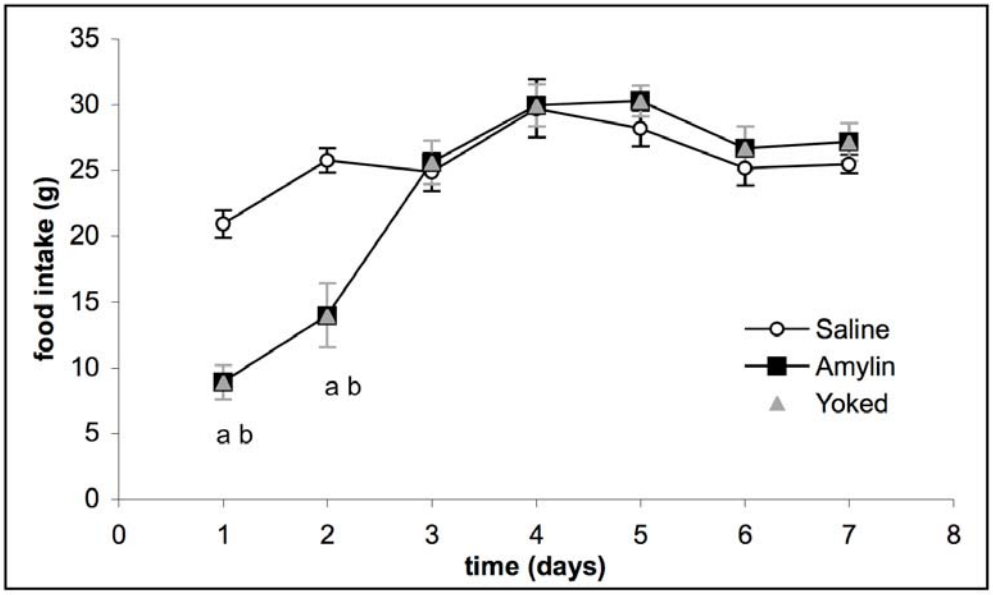

Figure 25: Effect of a chronic amylin infusion (6 µg/kg/h s.c.) on daily average food intake in rats (n = 8). Data are expressed as mean ± SE for every day. a: $p<0.05$ amylin vs. saline; b: $p<0.05$ yoked vs. saline (ANOVA one way).

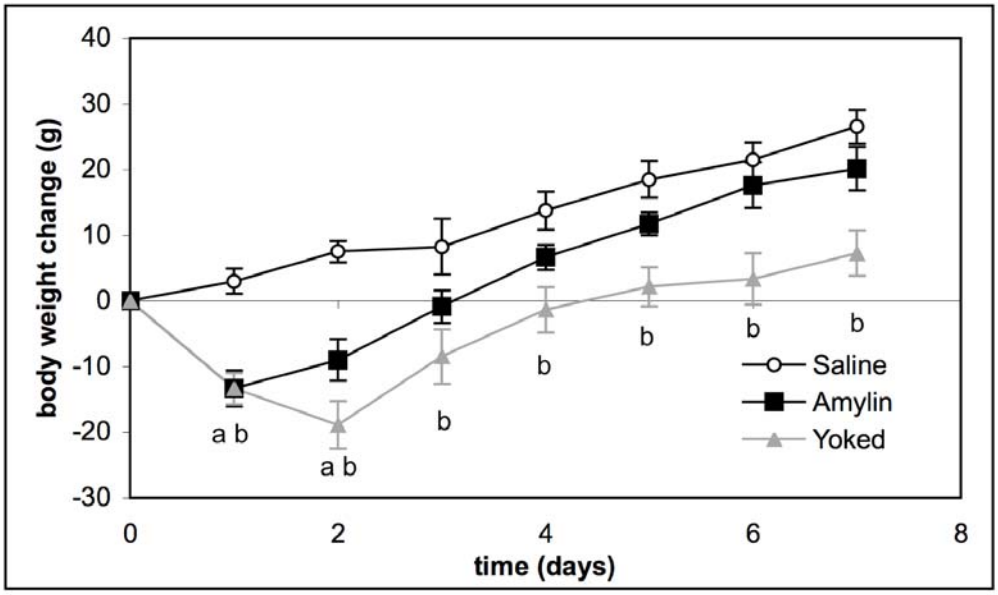

Figure 26: Effect of a chronic amylin (6 µg/kg/h s.c.) or saline infusion on body weight change in rats (n = 8). Data are expressed as mean ± SE for every day. a: $p<0.05$ amylin vs. saline; b: $p<0.05$ yoked vs. saline (ANOVA one way).

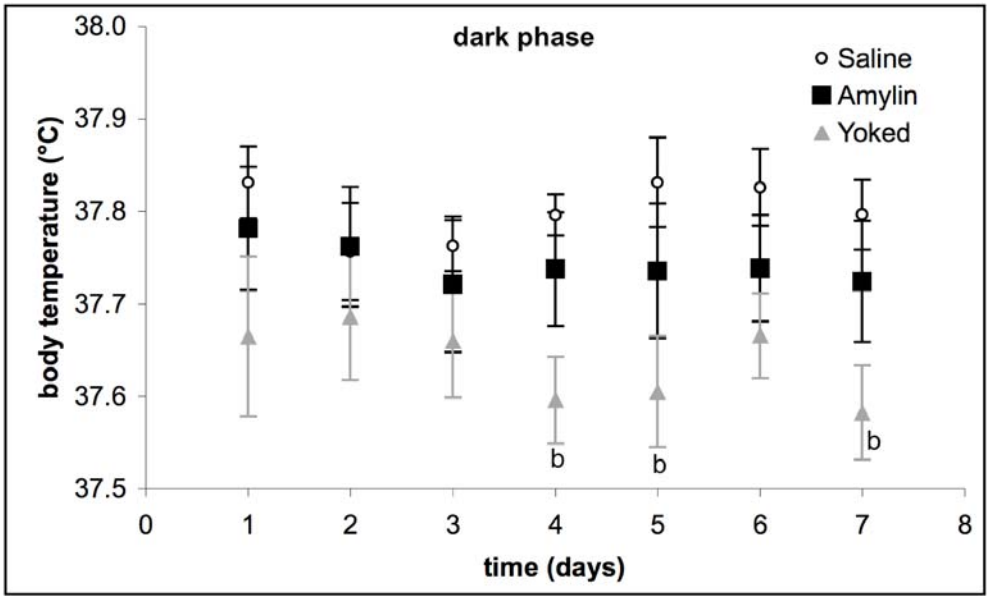

Figure 27: Average dark phase body temperature during chronic infusion of amylin (6 µg/kg/h) or saline in rats (n = 8). Data are expressed as mean ± SE for every day. b: $p<0.05$ yoked vs. saline (ANOVA one way).

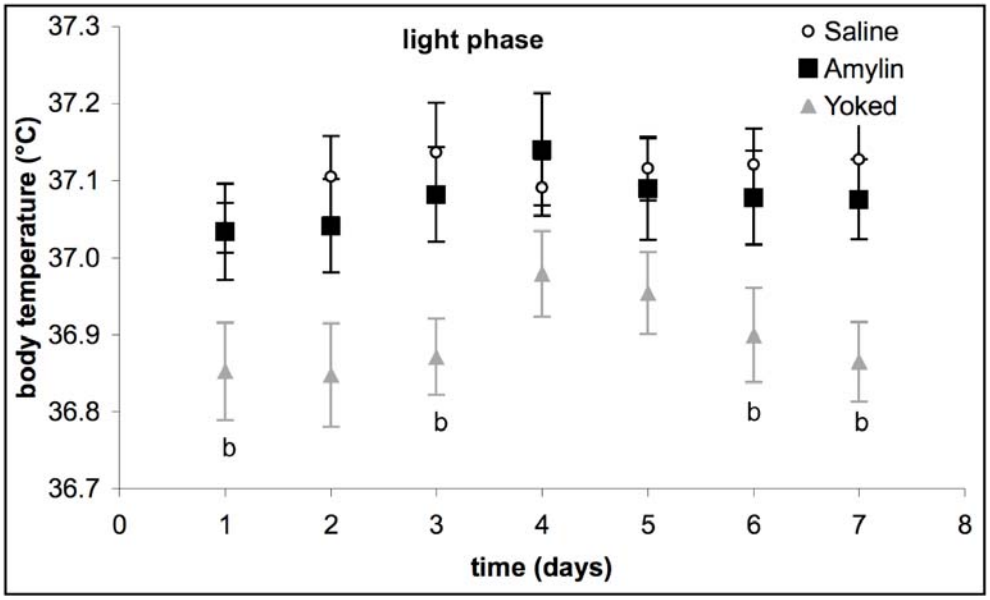

Figure 28: Average light phase body temperature during chronic infusion of amylin (6 µg/kg/h) or saline in rats (n = 8). Data are expressed as mean ± SE for every day. b: $p<0.05$ yoked vs. saline (ANOVA one way).

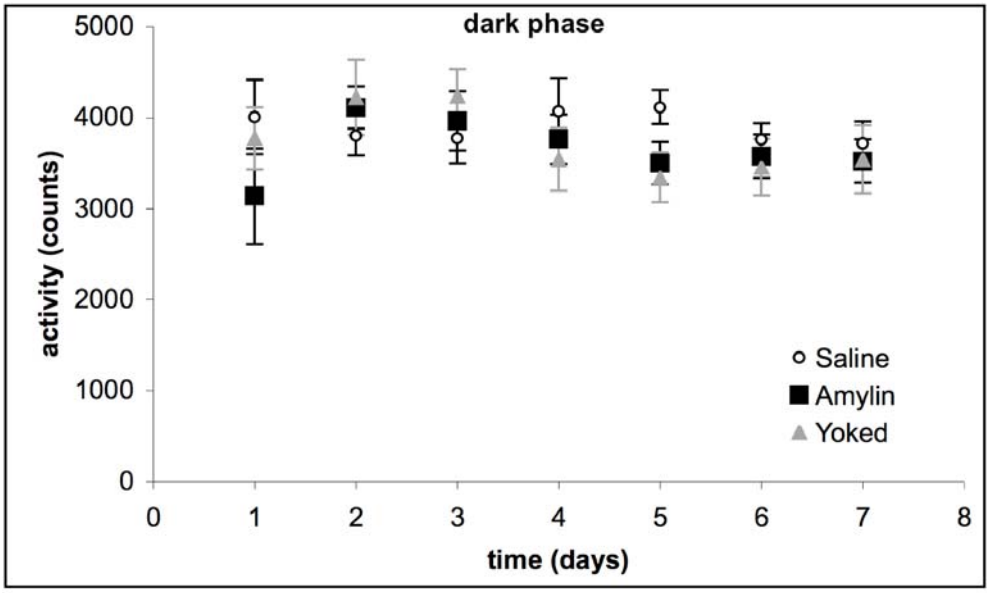

Figure 29: Average dark phase physical activity during chronic infusion of amylin (6 µg/kg/h) or saline in rats (n = 8). Data are expressed as mean ± SE for every day (ANOVA one way).

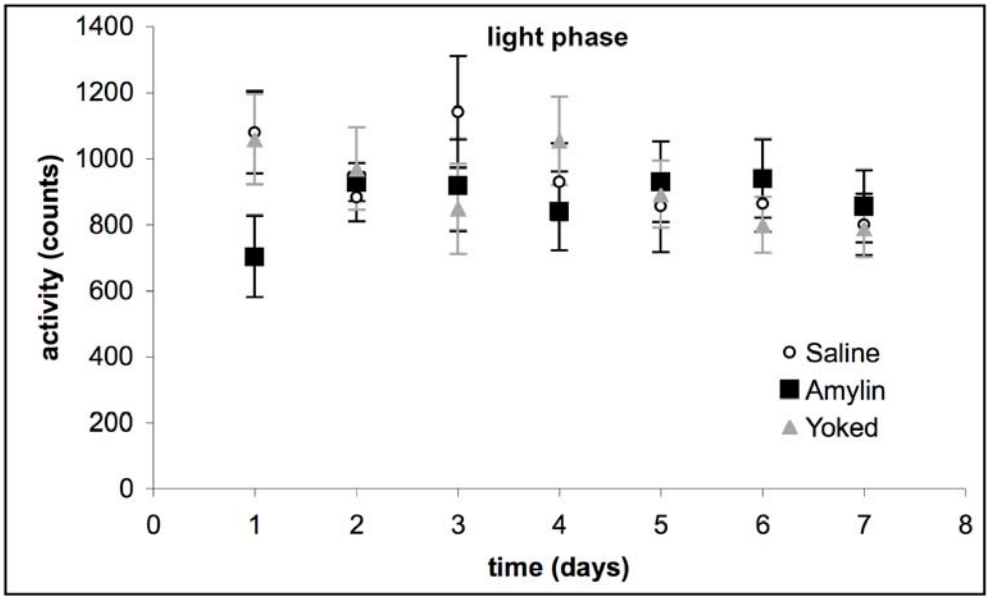

Figure 30: Average light phase physical activity during chronic infusion of amylin (6 µg/kg/h) or saline in rats (n = 8). Data are expressed as mean ± SE for every day (ANOVA one way).

6 Discussion

6.1 Summary of the results

A balance between energy intake and energy expenditure results in a constant body weight. The effect of amylin on food intake has been well investigated. However amylin's role in the control of energy output is not well established. The aim of the present study was therefore to investigate the acute and chronic effects of amylin and its agonist sCT on energy expenditure in rats. At a dose which significantly reduced food intake, a single injection of amylin did not significantly affect energy expenditure when injected in rats either at dark onset with ad libitum access to food, or when injected in the middle of the light phase without access to food. On the other hand, sCT, which has a more potent and long lasting effect on food intake due to its irreversible binding to the amylin receptor, significantly increased energy expenditure when rats had no access to food after a single injection. Refeeding resulted in a significant decrease of energy expenditure after an acute injection of sCT. This may have been due to the very strong reduction in feeding that was obvious at that time.

After a chronic infusion of amylin, energy expenditure tended to be increased compared to the yoked group but no effect was observed compared to the saline group. Body temperature was elevated in amylin infused rats, and they lost more body weight compared to the saline group. There was no effect on physical activity. Food intake was only reduced during the first days of chronic amylin treatment.

6.2 Effect of an acute amylin injection

A single injection of amylin (5 µg/kg) at dark onset had no effect on energy expenditure while food and water intake were significantly reduced 1h and 2h after

amylin administration. This reduction in food intake and water intake is consistent with previous studies (Lutz et al. 1994; Lutz et al. 1995b). Usually a reduction in food intake results in a decrease in energy expenditure (Passadore et al. 2004). Therefore our findings may indicate that amylin prevented the compensatory decrease in energy expenditure associated with a reduced food intake. This was also recently suggested by Mack et al. (2006) and Roth et al. (2006).

To exclude the possible confounding factor of amylin's effect on food intake, which might per se affect energy expenditure, rats were without access to food for 3h after the injection in the next experiment. Because rats are less active and hardly consume any food in the light phase, we performed the experiment in the middle of the light cycle. This was presumed to cause less stress as if rats had been fasted during their active night phase. Fasting during the light phase decreased this potential stress factor as much as possible (Del Prete et al. 1993).

The experiment was set up with three different doses of amylin (1, 5 and 10 µg/kg). Like in the first experiment amylin had no clear effect on energy expenditure. There was only a slight non-significant increase 30min after injection. It is possible that the short half-life of amylin (~ 13min [Young 2005] prevented a possible acute effect of amylin on energy expenditure after a single injection at a near-physiological dose. Because the air-tight cages had to be opened briefly just before the administration of amylin and because it took some time for a stable O_2/CO_2 measurement to be reached again it may well be that the equilibration of the atmosphere in the metabolic cages after injecting the animals might have taken too long for the recording system to detect a significant effect of amylin.

To circumvent the latter problem, rats were injected through a pre-implanted i.p. canula (see 4.8.3) in the next experiment. However, again no effect on energy expenditure was observed within the 3h after injection.

Because in some experiments the reported ED_{50} of amylin for food intake in rats is ~25 µg/kg, which is more than two times higher than the highest dose in our study, it cannot be excluded that the dose of amylin used in our experiments was too low to have a significant effect on energy expenditure (Young 2005).

6.3 Effect of an acute salmon calcitonin injection

Therefore, and because amylin at least tended to increase energy expenditure in food-restricted rats, we performed the next experiment with amylin's agonist salmon calcitonin. In fact a single injection of sCT (5 µg/kg) in the middle of the light phase in fasted rats resulted in a significant increase in energy expenditure. A possible explanation for the difference to the previous experiment with amylin is that sCT's effect is much longer lasting than amylin's effect due to an irreversible binding to the amylin receptor (Beaumont et al. 1993; Muff et al. 1995; Lutz et al. 2000). In parallel to the increase in energy expenditure, RQ was significantly elevated for the first 3h after the injection of the highest dose of sCT (5 µg/kg). This effect could be the result of some metabolic change, like a shift from fat oxidation to carbohydrate or protein oxidation. During fat oxidation RQ is about 0.7, during protein oxidation RQ is about 0.8 and during carbohydrate oxidation RQ is about 1.0. Usually RQ is the result of a mixed oxidation. Our results are not consistent with a study by Roth et al. They observed a decrease in RQ in amylin treated and pair-fed rats relative to vehicle controls, consistent with the decreased caloric intake in the former groups (Roth et al. 2006). The reason for the discrepant results is unclear.

After refeeding a significant decrease in energy expenditure and RQ were observed at the highest dose of sCT and, at least at some timepoints, also with the lower dose of 1 µg/kg sCT. These effects are probably caused by the strong reduction in food

intake, which may have overruled the direct effect on energy expenditure by sCT. During this period, the values of the RQ were close to 0.7, indicating a high lipid oxidation which is typical for animals that eat very little.

Similar to the previous experiments, no significant effect of sCT was observed on physical activity, suggesting that the increase in energy expenditure is caused by another mechanism. Roth et al. also observed that changes in spontaneous physical activity were not associated to the changes in energy expenditure (Roth et al. 2006). Other studies showed that doses of amylin that decrease food intake do not induce competing locomotor activities (i.e. hypo- or hyperactivity) (Roan et al. 2005). Upon central administration, high dose of amylin have been shown to decrease locomotor activity, an effect that would more likely be consistent with a reduction, rather than an enhancement, in energy expenditure (Clementi et al. 1996).

6.4 Effect of a chronic amylin infusion

Because amylin tended to increase energy expenditure after a single injection, and because sCT significantly increased energy expenditure after an acute injection, two additional experiments were performed with a chronic amylin infusion. In the experiment with the lower dose of amylin (2 µg/kg/h), energy expenditure was not significantly affected compared to the saline control group. However the yoked rats showed lower energy expenditure over the entire experimental period. This may suggest that there is in fact an effect of amylin on energy expenditure, because food intake was identical in the latter two groups and there was no effect on physical activity. Therefore, like in the acute studies, it seems that amylin prevented a decrease in energy expenditure that would typically be seen in animals that eat or

weigh less. The lower level of energy expenditure in pair-fed rats is consistent with a study of Roth et al. (2006).

Another indication that is consistent with this interpretation for an amylin induced effect on energy balance was observed in a study with amylin knockout-mice. These mice gained significantly more body weight compared to wildtype mice although cumulative food intake was not different, suggesting that energy expenditure was decreased in amylin knockout-mice (Gebre-Medhin et al. 1998; Lutz 2006).

In our study and despite the similar food intake in amylin-treated and yoked-fed rats, body weight loss was higher in the first days of amylin infusion. This is in line with Roth et al. They examined the body composition after a chronic amylin infusion (i.e, 300 µg/d) over 22 days, hence with a dose which is a two- or sixfold higher dose than what we used in our experiments. In Roth's study there was no difference in body weight change between amylin-treated rats and their pair-fed controls. Use of rats yoked to the amylin group is clearly advantageous to normal pair-feeding. Pair-fed rats receive the same amount of food, that was eaten by amylin treated rats on the day before. Yoked rats in our experiments received the same amount of food at the same timepoint as the control group. Therefore, because the amount of food that pair-fed rats receive is lower than the amount of food that they would eat under ad libitum feeding conditions, this "restricted" feeding may result in a fasting period at the end of the day. This is avoided with the yoked design. Roth et al. reported that carcass lipids tended to be reduced by simple caloric restriction, but only amylin-treated rats had a significantly lower percentage of fat and a significantly higher percentage of carcass protein, compared with the vehicle group. Body composition of pair-fed rats did not differ from that of vehicle-treated rats. Nuclear magnetic resonance analysis of body fat content before and after treatment showed that amylin and pair-feeding significantly reduced body fat relative to vehicle-treated rats.

However, amylin-treated rats lost significantly more fat than their pair-fed controls. Whereas amylin tended to slow the rate of gain of lean mass relative to vehicle controls, pair-feeding significantly reduced lean body mass. To examine whether there was regionally specific fat loss, selected fat depots were dissected and weighed. Amylin, but not pair feeding, significantly reduced retroperitoneal fat compared with vehicle controls (Roth et al. 2006). A specific decrease in epididymal fat pads after a chronic infusion was also observed in a study of Isaksson et al. These results confirm an effect of amylin on body composition although the mechanism is not completely investigated.

In our study amylin infusion resulted in an increased body temperature, especially in the light phase. This may explain the lack of decrease in energy expenditure, despite lower food intake. An effect of amylin on body temperature was also observed in a study by Bouali et al. They showed a dose-dependent increase in body temperature after acute central injections of amylin into the third ventricle (Bouali et al. 1995). The same effect was seen after a central injection into the PVN (Chance et al. 1992b). It should be noted, however, that these effects were seen after injections with much higher doses than we used in our studies. Although the mechanisms underlying the effect on body temperature under all these experimental conditions remains to be clarified, the presence of amylin immunoreactivity in the hypothalamus lends support to a possible role for amylin in thermoregulatory mechanisms (Chance et al. 1992b). It has been shown that amylin increases the concentration of serotonin in the hypothalamus (Chance et al. 1991; Chance et al. 1992b). Thus, it is possible that the hyperthermia induced by amylin is modulated via the release of serotonin because i.c.v. administration of serotonin is known to increase body temperature in animals (Blatteis 1981). Interestingly, however, amylin's anorectic effect is independent of the serotoninergic system.

The lower levels in energy expenditure, physical activity and food intake on day 1 compared to the following days are probably the result of the surgery on day 0. The main effects on body weight change, food intake and body temperature were mainly seen in the first days of the treatment, indicating that amylin was less effective after some days. Roth et al. also observed the main effects of amylin during the first week of their treatment period of 3 weeks. Similar to our studies they observed a strong decrease in food intake on days 1 to 4 and a body weight loss until day 3. On the following days body weight was stabilized (Roth et al. 2006). The strong reduction of food intake in the first days of a chronic amylin infusion is consistent with other studies (Arnelo et al. 1996; Isaksson et al. 2005).

Due to these promising effects of a low dose chronic amylin infusion the last experiment was performed with a higher dose of amylin (6 µg/kg/h). To avoid the strong effect of surgery on body weight seen in the previous experiment, temperature transmitters were already implanted a week before the start of the experiment and minipumps were implanted subcutaneously on day 0. In this experiment we observed a strong decrease of energy expenditure on the first day in the amylin and yoked groups. This was probably caused by a strong reduction in food intake, which overruled the direct effect on energy expenditure. On the following days, energy expenditure of the yoked fed rats was lower compared to the amylin group, although food intake was exactly the same. The reason for this is most probably the lower body temperature in yoked rats, because there was no difference in physical activity between groups. Body weight decrease on the first two days of this experiment was significantly stronger in amylin and yoked rats compared to the saline group. On the following days, however, yoked fed rats lost more body weight than amylin treated rats. This is in contrast to the first experiment using a lower dose of amylin and with the study of Roth et al. (Roth et al. 2006). Because there was no difference in

physical activity, food intake and water intake and body temperature was decreased, we do not have an explanation for this. If the yoked group were more stressed, due to restricted food availability, this would rather be expected to result in an increase in energy expenditure.

Overall we conclude that the experiment with the higher dose of amylin (6 µg/kg/h) was only more effective on food intake compared to the previous one (2 µg/kg/h). In both chronic experiments, the effect of amylin was relatively short-lasting, indicating that amylin was less effective after some days. Several mechanisms for amylin-induced desensitization have been proposed, including downregulation of the receptor or substrate consumption at the second messenger level (Furnsinn et al. 1993). Huang et al. observed a desensitization of amylin receptors in rat pancreatic acinar AR42J cells when they were pretreated with amylin but not with sCT or CGRP (Huang 1995). However, Roth et al. as well as Arnelo et al., observed a longer lasting effect of amylin with the same or higher amylin doses (Arnelo et al. 1996; Roth et al. 2006).

6.5 Final conclusions

In summary, a single bolus injection of a (near) physiological dose of amylin did not significantly influence energy expenditure when given at dark onset in rats that had ad libitum access to food or when given in the middle of the light phase to rats without access to food. However, the data indicate that amylin may prevent the decrease in energy expenditure that is normally seen in animals that eat less. Further an acute injection of sCT resulted in a significant increase in energy expenditure compared to controls when rats did not have access to food. This suggests that sCT had a stronger effect on energy expenditure than amylin itself, most likely due to its

irreversible binding to the amylin receptor. A chronic amylin infusion did not significantly influence energy expenditure compared to yoked rats and controls. Nevertheless, like in the acute studies, it seems that amylin tended to prevent a decrease in energy expenditure that would normally occur as a result of reduced food intake. This study shows that amylin may not only affect energy balance through its inhibitory effect on food intake, but also by influencing energy expenditure.

6.6 Future research

Several studies seem imperative to extend the knowledge on amylin's effect on energy expenditure. Because we only tested exogenous amylin it would be important to perform an experiment with amylin's antagonist AC187 which should block the action of endogenous amylin. Administration of this antagonist is expected to result in a decrease in energy expenditure.

Further we plan to perform an experiment with amylin knockout-mice. Because they gained more body weight compared to wildtype mice although food intake was similar, it would be expected that energy expenditure was reduced in the knockout-mice. Further we expect that the effect can be rescued when replacing amylin.

To avoid amylin desensitization in a chronic setup as possibly observed here, amylin could be infused intermittently e.g. only in the dark phase or only in the light phase. This could be performed with minipumps connected to a tubing, alternately filled with amylin and a control solution.

Finally, a study with central amylin infusions may probably affect energy expenditure stronger than an i.p. injection because an effect on food intake after central administration also occurs at lower doses than when given peripherally.

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